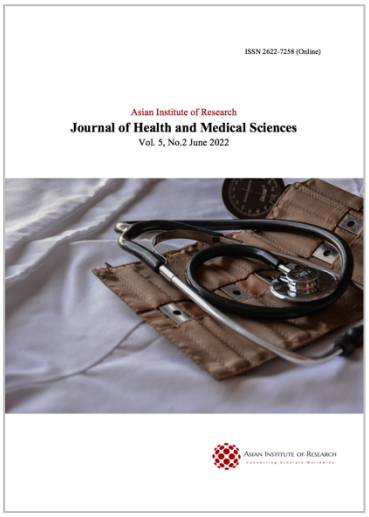

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DNA Cell Cycle Analysis with Propidium Iodide (PI) in Liver Cancer Patients

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Abstract
Hepatocellular Carcinoma (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 chronic liver injury 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). Biomarkers are being developed as alternatives to liver biopsy for predicting liver fibrosis in patients with chronic hepatitis C. The aim of this study was to investigate DNA Cell cycle in HCC, Fibrosis patients and compare with healthy control group. They were classified into three different groups 51 individuals diagnosed Fibrosis patients, 30 Hepatocellular Carcinoma patients and 40 healthy Control individuals with no liver diseases. Tumor Markers including CEA and AFP were estimated using ELISA method, DNA cell cycle was assessed using Flow Cytometry. Results showing sub G1 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 ($p<0.005$). On the other hand, G0/G1 was decreased significantly in both HCC and Fibrosis patients with values 62.2 ± 10.02 and 53.4 ± 11.1, respectively while in Healthy control. In addition, AFP and CEA were increased in both HCC and Fibrosis patients comparing with Control. Conclusion based on this study DNA cell cycle could help in assessment of Liver damage especially HCC.

Keywords: Hepatocellular Carcinoma, HCV, NASH

1. Introduction

Liver diseases affect the normal functions of the liver causing decrease in its performance. Abnormalities in the liver functions, however, are usually not apparent in most individuals with chronic liver disease until the disease is rather advanced. Liver diseases could be classified into infectious (e.g., viral hepatitis), toxic (e.g., alcohol-related diseases), genetic (e.g., hemochromatosis), immune (e.g., autoimmune hepatitis primary biliary cirrhosis), and neoplastic (e.g., hepatocellular carcinoma) Dufour et al. (2000). Hepatitis is inflammation of the liver. Viral infection is responsible for around half of all cases of acute hepatitis. The term is generally used to refer to the diseases caused by viruses including the diseases hepatitis A–E, and disease due to cytomegalovirus, Epstein-Barr virus, adenovirus, rarely herpes simplex virus and others Akiba et al. (2005). Only hepatitis B virus and hepatitis

C virus are able to persist in the host and cause chronic hepatitis Herzer et al. (2007). Hepatitis C virus is also a major public health problem Michielsen et al. (2005). Chronic hepatitis is defined as ongoing hepatic necrosis and inflammation of the liver, often accompanied by fibrosis. Chronic hepatic injury may progress to cirrhosis (15-20 % in the case of chronic hepatitis C virus) and predisposes to hepatocellular carcinoma. Most commonly, it is the result of chronic viral infection. Chronic hepatic injury is a relatively common disorder with minimal symptoms but long-term risk of significant morbidity and mortality Michalopoulos et al. (2010). Hepatocellular carcinoma (HCC) is a primary malignancy of the liver and occurs predominantly in patients with underlying chronic liver disease and cirrhosis. The cell(s) of origin are believed to be the hepatic stem cells, although this remains the subject of investigation. Tumors progress with local expansion, intrahepatic spread, and distant metastases Hashimoto et al. (2004).

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. G1 also known, as gap-phase 1 is the period during which cells prepare for the process of DNA replication. S-phase is defined as the stage in which DNA synthesis occurs. G2 is the second gap-phase during which the cell prepares for the process of division and M stands for mitosis, the phase in which the replicated chromosomes are segregated into separate nuclei and cytokinesis occurs to form two daughter cells. In addition to G1, S, G2, and M, the term G0 is used to describe cells that have exited the cell cycle and become quiescent (Johnson and Walker 1999). The liver is exposed to insults from external factors that can induce hepatocyte cell death and liver mass loss, triggering liver regeneration. During this process, which depends on reactivation of transcriptional program leading to exit from quiescence and to enter into the cell cycle of (mostly) hepatocytes, the liver maintains all its metabolic functions Sumpter et al. (2005)

2. Subjects and Methods

This study Carried on 121 individuals they were classified into three different groups: Group (I): 51 individuals diagnosed Fibrosis patients they were, 42 males and 9 females, their age ranged from 43-87 year with a mean of 59.9±10.1 year. Group (II): 30 Hepatocellular Carcinoma patients, 23 males and 7 females, and their age ranged from 43-74 year with a mean of 57±8.81 year. Group (III): 40 healthy Control individuals with no liver diseases 28 males and 12 females, and their age ranged from 20-51 year with a mean of 30.8±7.57 year. Patients with clinically and laboratory confirmed chronic hepatitis C (CHC) were included in the present study; other causes of chronic liver disease were ruled out. They were recruited from Gastroenterology Surgical Center, Mansoura University, Egypt, that approved the present study. An informed consent was obtained from each individual participated in the present study and all were fully informed concerning the nature of the disease and the diagnostic procedures involved. No patient had received interferon treatment before liver biopsy and blood collection. Patients with reduced production of platelets other than hepatic infection with HCV such as infection of typhoid, deficiency of vitamin B12 and leukemia were excluded from the study.

The HCV infection classification of the three groups was diagnosed based on biochemical, serologic, and histological criteria. None of the patients had history of habitual alcohol consumption or hepatocellular carcinoma. Moreover, all individuals were positive for anti-HCV antibody and were negative for hepatitis A and B viruses testing. All patients were negative test for anti-HIV antibodies. In addition to 40 samples were obtained from healthy individuals. Blood samples were collected from all patients by vein-puncture within 2 weeks of liver biopsy and a part of the blood was treated immediately with EDTA-K2. Routine blood pictures including platelet counting were determined by KX-21 Sysmex automated hematology analyzer (Sysmex Corporation, Japan). Sera were separated from the rest of blood samples and tested fresh for liver function indexes.

Sera were processed for the biochemical analysis of Albumin, Bilirubin, Aspartate aminotransferase (AST) and Alanine aminotransferase (ALT) according to the manufacture instructions. All serum samples were obtained with informed consent. The AST-ALT ratio was calculated as [AST/ ALT].

Aspartate Platelet Ratio Index (APRI): was calculated according to Wai et al. (2003) as the following equation: (AST [IU/L] / upper limit of normal AST [IU/L]) × 100 / platelet count [109/L].

Fibrosis-4 index (FIB-4): was calculated according to Sterling et al. (2006) as the following equation: AST [IU/L] × age [years]/ platelet count [109/L] × ALT [IU/L] 1/2

Estimation of Alpha Fetoprotein (AFP): AFP was estimated according to method of Silver et al. (1973). Based on the principle of a solid phase enzyme-linked immunosorbent assay. The assay system utilizes a rabbit anti-AFP antibody directed against intact AFP for solid phase immobilization (on the microtiter wells).

Quantitative Determination of Carcinoembryonic Antigen (CEA): CEA was estimated according to method of Hansen et al. (1989). The CEA Quantitative Test Kit is based on a solid phase enzyme-linked immunosorbent assay. The assay system utilizes one monoclonal anti-CEA antibody for solid phase (microtiter wells) immobilization and another mouse monoclonal anti-CEA antibody in the antibody-enzyme (horseradish peroxidase) conjugate solution.

Flow Cytometry Technique for Evaluation of DNA Cell Cycle. Peripheral Blood Mononuclear Cell (PBMC) Isolation and Red Cell Lysis Procedures: PBMC isolation: Human PBMC are isolated using a density gradient technique. The two most commonly used density gradient solutions are Ficoll-Paque PLUS (Sigma-Aldrich, St. Louis, MO, United States). DNA Cell Cycle Analysis with Propidium Iodide (PI): Propidium Iodide: The most commonly used dye for DNA content/cell cycle analysis is (PI). It can be used to stain whole cells or isolated nuclei. The flow cytometer must be equipped with an argon laser with an optimal emission of 488 nm. Primary emission of propidium iodide is between 580 and 650 nm Vindelov et al. (1983). key phases of the cell cycle may be defined: G0/G1 = Phase in which the cell is resting or preparing for mitosis. S-Phase = Phase between G0/G1 and G2/M when the cell is in the process of DNA synthesis. G2/M = Phase in which the DNA content of the cell is twice the amount of the DNA content of the cell in the resting phase.

Statistics

A computer software package SPSS was used in the analysis. For quantitative variables, mean and median, standard deviation. Frequency. ANOVA test was used for comparing between different groups.

3. Results

Table 1: Clinical Parameters in all groups

Clinical Parameters Mean ± Sd	Fibrosis	HCC	Healthy Control	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<0.005
Bilirubin (mg/dl)	4.1 ± 6.3	7.4 ± 11.0	0.3 ± 0.18	P<0.005
Platelets ($\times 10^9$)	200.8 ± 47.9	153.8 ± 54.5	289.3 ± 16.6	P= 0.000
AST/ALT Score	1.4 ± 0.7	1.5 ± 0.58	1.07 ± 0.37	P<0.005
APRI Score	1.3 ± 1.3	0.98 ± 0.61	0.1 ± 0.2	P<0.005
FIB-4 Score	4.2 ± 3.6	3.7 ± 2.3	0.38 ± 0.13	P<0.005
Albumin (g/dl)	3.2 ± 0.15	2.91 ± 0.25	4.3 ± 0.43	P<0.005

P > 0.05 is considered not significant. P < 0.05 considered significant < 0.001 considered very significant. P < 0.0001 is considered extremely significant

Table 2: Tumor Markers Parameters in all groups

Tumor Markers Parameters	Fibrosis	HCC	Healthy Control	P-value
Alpha FetoProtein (ng/ml) Mean ± SD	25.08 ± 22.2	423.3 ± 4.7	1.68±0.74	P < 0.001
Carcinoembryonic Antigen (µg/l) Mean ± SD	2.64 ± 0.66	3.61 ± 0.6	1.26±0.13	P < 0.005

P > 0.05 is considered not significant. P < 0.05 considered significant < 0.001 considered very significant. P < 0.0001 is considered extremely significant

Table 3: DNA Cell Cycle in all groups

DNA Cell Cycle	Fibrosis	HCC	Healthy Control	P-value
Sub G1 phase	17.7 ± 5.9	9.55 ± 4.72	5.7 ± 3.36	P = 0.000
G0/G1 phase	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
G2/M phase	18.3 ± 11.3	10.2 ± 7.23	2.4 ± 0.81	P <0.005

P > 0.05 is considered not significant. P < 0.05 considered significant < 0.001 considered very significant. P < 0.0001 is considered extremely significant

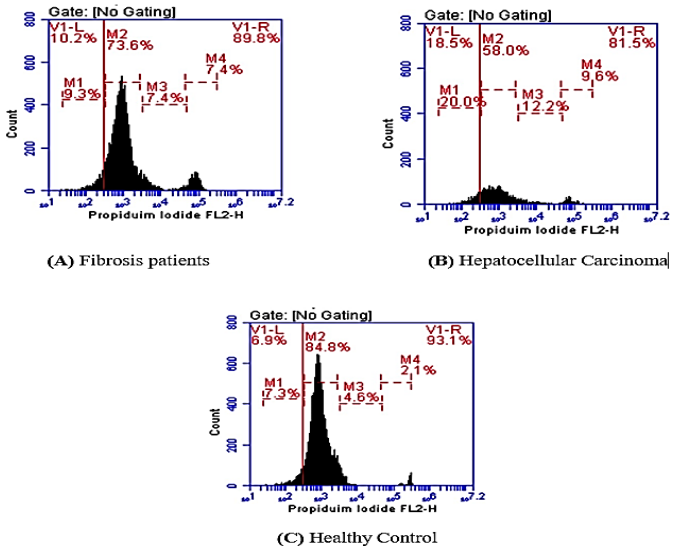

Figure 1: Flow Cytometry of DNA cell cycle using PI

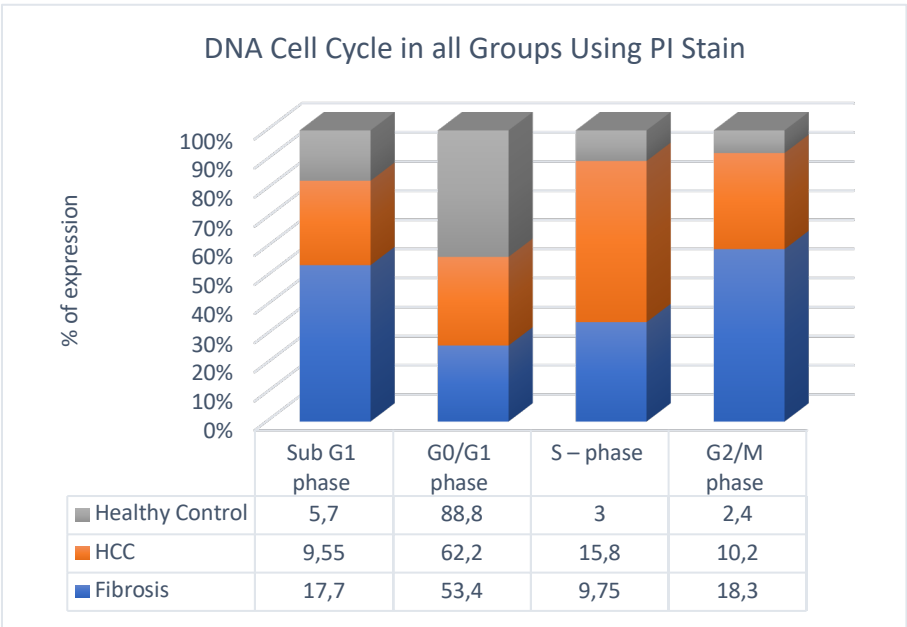

Figure 2: DNA Cell cycle in all groups

4. Discussion

Egypt is the highest HCV prevalence country in the world; in 2008, the Egyptian Demographic Health Survey (EDHS), which was conducted on a large nationally representative sample, estimated the prevalence of HCV antibodies and HCV RNA, among the 15–59-year age group, to be 14.7 and 9.8% respectively. Based on the population census and the EDHS done in 2008, it was estimated that more than 6.8 million persons aged 15–59 years had HCV antibodies, of which more than 4.5 million individuals had active HCV infection (El-Zanaty and Way 2009). Hepatocytes in the liver are the predominant targets of HCV infection, and the entry of HCV into hepatocytes is a stringently coordinated process that relies on successive and concerted interactions between the envelope glycoproteins E1 and E2 and host cellular factors that are present on the cell surface, i.e., the so called "entry (co)receptors" The known (co)receptors, including the tetraspanin CD81 (Pileri et al. 1998; Zhang et al. 2004). 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 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; 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). The elevated aminotransferase value in HCC reflects damage to adjacent hepatocytes as a direct result of tumor growth or damage to more remote liver cells caused by interference with their blood supply or venous drainage. It may also be due to continuing liver cell necrosis in those with concomitant active cirrhosis or chronic active hepatitis Domingo et al. (2013).

Out of 30 patients 13 HCC patients showed AFP level below 200 ng/ml. In our study, AFP was significantly higher in HCC patients compared to Fibrosis group. An elevated AFP (> 400 ng/mL) level was associated with advanced disease stage in HCC patients. Some previous studies showed that AFP has a low specificity for diagnosing HCC to the extent that the American Association for the Study of Liver Diseases-Practice Guidelines Committee has recently recommended ultrasound examination alone (without AFP) to be used for HCC surveillance Massoud et al. (2006). However, the interpretation of ultrasound is operator dependent and can be difficult in persons who are obese or have underlying cirrhosis. Therefore, other reliable biomarkers are required to complement ultrasound and AFP for proper diagnosis and early detection of HCC Lok et al. (2011). In the present study AFP was estimated in Fibrosis, Hepatocellular Carcinoma and Healthy Control using ELISA, the Mean values of AFP were in Fibrosis, HCC patients and control were 25.08 ± 22.2 and 423.3 ± 4.7 and 1.68±0.74; respectively (p<0.001). Carcinoembryonic antigen (CEA) is a high-molecular-weight glycoprotein (180–200 kDa) consisting of a 60% carbohydrate composition. Normally, serum CEA concentrations are below 5 ng/mL. In contrast to healthy conditions, elevated serum CEA levels have been found in other types of cancers aside from colorectal cancer, to include gastric, pancreatic, breast, and genitourinary cancers Tangkijvanich et al. (2000). Cell cycle begins from the completion of one division to the end of the next, leading to the generation of two daughter cells. Mammalian cell cycle is tightly regulated and can be artificially divided into four distinct phases (G1, S, G2, and M) according to their specific characteristics (Martínez-Alonso et al. 2019; Martínez-Alonso and Malumbres 2020) G1 phase is the gap phase, which is characterized by cell growth in size and the synthesis of RNAs and proteins required for DNA duplication. S phase is the synthesis phase during which DNA is synthesized. G2 phase is another gap phase, in which stage cells are characterized by rapid growth in cell size, more protein synthesis and preparation for division. M phase is the mitosis phase, during which the replicated chromosomes are segregated into separate nuclei and cytokinesis promoting the formation of two daughter cells. At the end of the M phase, 1 cell divides into two daughter cells, each of which contains one copy genomic DNA of the mother cell, and a cell cycle is accomplished.

Sub G1 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). On the other hand, G0/G1 was decreased significantly in both HCC and Fibrosis patients with values 62.2 ± 10.02 and 53.4 ± 11.1; respectively while in Healthy control group G0/G1 was 88.8 ± 3.32 (p<0.005). Our data revealed that S-phase was increased significantly in both HCC and Fibrotic patients with Mean ± SD. 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 G2/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 G2/M phase was 2.4 ± 0.81 ($p<0.005$).

5. Conclusion

Based on this study, liver exposed to insults from external factors can induce hepatocyte cell death and liver mass loss, triggering liver regeneration. HCC causing increasing in S-Phase in DNA cell cycle comparing with fibrosis and healthy control which could help in assessment of Liver damage.

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