

Measurement of the Gene Expression and Polymorphisms of c-myc and p53 Genes in HBV Infected Patients

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Abstract: This study explained the c-myc and p53 gene expression and polymorphisms in chronic (40), cirrhosis (30) and Hepatocellular Carcinoma, (HCC) (30) patients related to Hepatitis B Virus (HBV) infection and healthy control (50) in Egypt. Where, c-myc (intron 8) and p53 (codon 72) gene expression and polymorphisms were determined using qRT-PCR and PCR-RFLP techniques. The results showed that c-myc gene expression ($2^{-\Delta\Delta Ct}$) was significantly increased in chronic (1.38), cirrhosis (1.47) and HCC (5.59) compared to the control group (1.00), while p53 gene expression ($2^{-\Delta\Delta Ct}$) was significantly decreased compared to the control group (0.82, 0.65, 0.33 and 1.00, respectively). In HCC group, c-myc genotype (CC) was predominant (90%) more than cirrhosis, chronic and control (73.33, 22.5 and 6%, respectively), GG genotype was predominant in control (70%) more than chronic, cirrhosis and HCC groups (67.5, 6.66 and 6.66%, respectively) and GC genotype was high in control (24%) more than cirrhosis, chronic and HCC groups (20, 10 and 3.33%, respectively). p53 PP (88%) genotype was predominant in control more than chronic, cirrhosis and HCC groups (30, 30 and 6.66%, respectively), AA genotype was predominant in HCC group (73.33%) more than chronic, cirrhosis and control (50, 10 and 4%, respectively) and genotype PA was predominant in cirrhosis (60%) more than chronic, HCC and control (20, 20 and 8%, respectively). These results suggest clearly that both c-myc and p53 gene expression and polymorphisms influence clinical outcome and progression of HBV infection and then considered an accurate genetic biomarker to determine and predict the progression of HBV infection.

Keywords: HBV, c-myc, p53, Gene Expression, Genetic Polymorphism

Introduction

Worldwide, chronic hepatitis, cirrhosis and Hepatocellular Carcinoma (HCC) are mainly caused by Hepatitis B Virus (HBV). HBV disease ranks the 10th among leading to causes of death (one million/year) (MacLachlan and Cowie, 2015). By the necroinflammatory process including inflammation and liver regeneration, chronic HBV infection was characterized (Hsieh *et al.*, 2011; Mah *et al.*, 2011). In all over the world, more than 400 million persons are chronically infected with HBV, which is responsible for more than 300 thousand cases of HCC per year (Lai *et al.*, 2003). Consequently, HBV infection considers a global health problem and a major risk factor for HCC (Bréchet, 2004). Many factors are known for affecting

the clinical results of chronic viral hepatitis, inclusive: Viral, host, environmental and genetic factors (Kao and Chen, 2005). Genetic polymorphisms of both p53 and c-myc genes have been associated to the development of HCC, although, their impacts remain controversial on the progression of liver disease (Poeta *et al.*, 2007).

p53 tumor suppressor protein has a well established role in repression of the cancer (Levine and Oren, 2009). Predominantly, tumor suppressive activities of this protein can be conferred by regulation as a transcription factor, in addition the transactivating over or more than 200 different target genes. p53 was also found as critical factor prevailing innate and acclimatize aging, development, immune responses, neural degeneration and reproduction (Chang *et al.*, 2012; Menendez *et al.*, 2013). Arg72Pro is one of the familiar polymorphisms of

the p53 gene in codon 72 (Ex4+119 G>C; rs1042522), encoding an arginine/proline substitution of the p53 protein. So, two polymorphic variants of this protein were shown having some difference in biochemical and biological properties (Dumont *et al.*, 2003). A report mentioned that Arginine variant induces apoptosis more effective than Pro variant, which may influence cancer risk. Pro variant was identified as a risk factor of some malignancies containing HCC, while the relationship between these polymorphisms and the outcome of HBV infection was not firmly established (Yoon *et al.*, 2008).

C-myc protooncogene is the human cellular homologue of the v-myc oncogene of avian retrovirus MC29 (myelocytomatosis), which is located at chromosome 8 (8q24.12-q24.13). Croce *et al.* (1983) found that c-myc acts as a strong factor for the transcription process, in addition, implicated in both the control of cell differentiation and apoptosis (Croce *et al.*, 1983). By the investigation of Asn11Ser polymorphisms, this locus was assigned and located within the N-terminal domain of c-myc (pfam 01056), which was away from the conserved c-myc Box I (35 amino acids). C-myc physiological function is the promotion of cell cycle progression during the activation of genes, such as, CCND2 which encodes both CDK2 and cyclin D2 (Hermeking *et al.*, 2000). Additionally, c-myc plays an important role in the cell growth, as a result of the activation of both translation initiation factors eIF2a and eIF4E (Coller *et al.*, 2000) and inhibits cell differentiation (Grandori *et al.*, 2000). Moreover, c-myc is active in 70%, approximately, in all human cancers (Nilsson and Cleveland, 2003).

In Egyptian population, we study the effects of both c-myc and p53 gene expression and genetic polymorphisms on the progression of HBV infection. Where, we determine the putative association of both c-myc and p53 gene expression, Asn11Ser polymorphisms in both c-myc and p53 Arg72Pro polymorphisms of HBV infection (chronic, cirrhotic and HCC patients) in different stages, using both qRT-PCR and PCR-RFLP techniques.

Materials and Methods

Patients and Samples Collection

In the present study, all patients gave their informed consents for inclusion before participating in this study. This study was conducted in conformity with Helsinki's Declaration; also the protocol was approved by the Ethics Committee of Faculty of Medicine, Alexandria University, Egypt. This study was started from April, 2015 to December, 2016. 150 patients with liver diseases cases were attended to hepatology and gastroenterology Department, Faculty of Medicine, Alexandria University, Alexandria, Egypt. According to (Neuveut *et al.*, 2010), the patients were diagnosed based clinical, biochemical,

serological information, serum Alpha/Fetoprotein level (AFP) and abdominal ultra-sonographic findings, which supplemented by histological examinations. While, HCC diagnosis was based the positive findings of cytological or pathological examination, the positive images on angiogram, the ultrasonography, the computed tomography and the combined with a-fetoprotein level (more than 200 ng/mL) (Sheen *et al.*, 2003). According to the previous diagnosis, the patients are classified into three main groups. Group I: 40 Patients with chronic HBV infection (determined as positive to Hepatitis B surface Antigen (HBsAg) for more than six months). Group II: 30 Patients with cirrhotic liver due to HBV infection. The cirrhotic patients have been diagnosed by B-Scan Ultrasonography (USG), where, irregularity of the hepatic surface, inhomogeneity of the hepatic tissue and enlargement of the caudate lobe were appeared. Many biochemical indices were carried out, such as thrombocytopenia, along with impaired hepatic biosynthesis (low concentrations of both albumin and cholinesterase, in addition, an altitude of the International Normalized Ratio (INR)). The impairment of the detoxifying function from the liver (elevated bilirubin concentration) and transaminase concentrations (AST and ALT) were, generally, in the normal range or mildly elevated. Group III: 30 Patients with Hepatocellular Carcinoma (HCC) due to HBV infection. The fourth group (control) contains 50 HBV negative individuals (33 males and 17 females). Fifty patients were excluded from this study due to coinfection with Hepatitis A Virus (HAV), Hepatitis C Virus (HCV), hepatitis delta virus (HDV), Hepatitis E Virus (HEV) and Human Immunodeficiency Virus (HIV), additionally, hepatorenal syndrome (as there are a strong correlation between hepatorenal syndrome and genetic expression of c-myc and p53). The patients with history of schistosomiasis infection, heavy alcohol abuse, NAFLD, NASH or other genetic liver diseases have been excluded to not interfere with the association between HBV infection only and the genetic expression, polymorphism of c-myc and p53. Five ml Blood sample was obtained from each participant and then sera were separated and stored at -80°C until tested.

Biochemical and Serological Tests

A serum for each sample was distributed into three parts. The first part was used for liver biochemical tests, such as, alanine Aminotransferase (ALT), Alfa Fetoprotein (AFP), aspartate Aminotransferase (AST), Gamma Glutamyl Transferase (GGT), serum albumin and total serum bilirubin level (Spinreact, England). The second part was used for HBV infection serological markers, such as, Hepatitis B core IgG antibody (anti-HBc IgG), Hepatitis B core IgM Antibody (anti-HBc IgM), Hepatitis B envelope Antibody (anti-HBe), Hepatitis B envelope Antigen (HBeAg), Hepatitis B

surface antibody (anti-HBs), Hepatitis B surface Antigen (HBsAg), total Hepatitis B core Antigen (HBcAg). All HBV serological markers were purchased from (Bio-Rad, France except anti-HBc IgG from ALPCO DIAGNOSTIC, USA). Finally, the third sera part was frozen at -80°C for further molecular assays, such as genomic DNA and RNA extraction, HBV DNA extraction, quantitative HBV DNA titres and c-myc and p53 genetic polymorphisms.

Genomic DNA, Genomic RNA Extraction, HBV DNA Extraction and Quantification

From 100 μL of sera samples, genomic DNA was extracted using DNA isolation kit (Genomic, Bioline, England). In distilled de-ionized water (ddH_2O), DNA samples were dissolved and kept at -20°C till used for c-myc and p53 genes polymorphism by using PCR and RFLP techniques. While, from 150 μL of sera samples, total RNAs was extracted using RNA mini kit (Bioline, England). RNA samples were dissolved in 70 μL elution buffer and kept at -20°C until using for c-myc and p53 gene expressions by quantitative Real Time PCR (q-RT-PCR) assay. According to (Weston *et al.*, 1997), sample from each patient was tested for quantitative HBV-DNA using real-time PCR technique. By Cobas AmpliPrep instrument (Roche Diagnostics Limited, Rotkreuz Switzerland), from 850 μL of serum, HBV-DNA was extracted. Cobas TaqMan 48 Analyzer was used for both automated real-time PCR amplification and detection of PCR products. It should be noted that, HBV-DNA levels were expressed in IU/ml.

qRT-PCR of c-myc and p53

From sera samples, genomic RNA was extracted and used as template to examine the expression level of both c-myc and p53 genes in the existence of housekeeping gene primers (GPDH). Primer sequences were as follows: c-myc sense: 5'-TGCCTCCCGCTTTGTGTG-3', antisense: 5'-CATTCCCAGCACCTCCTATT-3'; p53 sense: 5'-AGGGATACTATTCAGCCCGAGGTG-3', antisense: 5'-ACTGCCACTCCTTGCCCCATTC-3' and GAPDH sense: 5'-ATTGACCACTACCTGGGCAA-3', antisense: 5'-GAGATACACTTCAACACTTTGACCT-3'. qRT-PCR reaction mixture was accomplished in 25 μL , containing 12.5 μL 2X Quantitech SYBR Green RT Mix (Fermentaz, USA), 2 μL extracted RNA (50 $\text{ng}/\mu\text{L}$), 1 μL of both forward and reverse primers (25 $\text{pmol}/\mu\text{L}$), 9.5 μL RNase (free water). The program was performed as follows: Initial denaturation at 95°C for 10 min, 40 cycles of 95°C for 15 sec, annealing at 60°C for c-myc and 64°C for p53 for 30 sec and extension at 72°C for 30 sec. Data acquisition was performed during the extension

step, while the reaction was performed by Rotor/Gene-6000 system (Qiagen, USA). By Rotor/Gene-6000 Series Software, comparative quantitation analysis was done as well. The data set of both samples and control of real-time PCR was analyzed with appropriate bioinformatics and statistical program for estimation of the relative expression of genes using real time PCR and the results normalized to GPDH gene (Reference gene). Statistically, the data were interpreted, evaluated, analyzed by Rotor/Gene-6000 (version 1.7) and expressed as ($2^{-\text{ddct}}$).

Amplification and Genotyping of c-myc and p53 Genes by PCR-RFLP

For amplification of c-myc and p53 genes, primer sequences used for specific PCR were as follows: c-myc forward: 5'-CAGTTGTCCCTGCCTTTTCT-3', c-myc reverse: 5'-CATTCCCAGCACCTCCTATT-3'; p53 forward: 5'-ATCTACAGTCCCCCTTGCCG-3', p53 reverse: 5'-GCAACTG ACCGTGCAAGTCA-3' (Olivier *et al.*, 2009; Litviakov *et al.*, 2010). PCR was done in 20 μL reaction volume containing 2.5 μL genomic DNA (25 ng), 2 μL of each primer pair (0.2 pmol), 2.5 μL dNTPs (200 nM), 1 μL Taq DNA polymerase (1 $\text{u}/\mu\text{L}$), 2.5 μL PCR reaction buffer (2X) and 7.5 μL ddH_2O . Thermal cycling (MyGene Series Peltier Thermal Cycler) condition of the mixture included denaturation at 94°C for 5 min, 35 cycles of PCR amplification conducted for 30 sec at different annealing temperatures for each primer set (60°C for c-myc and 56°C for p53) with primer extension at 72°C for 40 sec and final extension was performed at 72°C for 5 min. The amplified products were conducted on 2% agarose gel electrophoresis, then stained with ethidium bromide.

For restriction analysis of the amplified c-myc and p53 genes, 10 μL of each PCR product (681 bp and 296 bp, respectively) was accomplished with 10 units HhaI and Bsh1236I restriction enzymes, respectively (Fermentas Life Science, Germany) for 24 h at 37°C (Litviakov *et al.*, 2010; Olivier *et al.*, 2009). Digested DNA was examined on 3% agarose gel buffer (0.5X TBE), stained using ethidium bromide, visualized under UV light and then photographed by Gel Documentation System (Alpha Imager MI220, Documentation and Analysis System, Canada).

Statistical Analysis

Serological, biochemical and molecular data were Statistically Analyzed by SAS 9.1 program (SAS, USA). The results were displayed as percentages for both categorical variables and mean with Standard Deviation (SD) for continuous variables. Statistically, P value ≤ 0.05 was considered significant.

Results

General Characteristics of the Studied Groups

Four groups were included in the current study. Group I (chronic HBV) contains 40 persons (70% males and 30% females), their ages ranged from 27 to 42 years, mean \pm SD (39.6 \pm 12.3 years). Group II (cirrhosis HBV) contains 30 patients (70% males and 30% females), their ages ranged from 29 to 55 years, mean \pm SD (42.3 \pm 13.8 years). Group III (HCC related to HBV) contains 30 patients (80% males and 20% females), their ages ranged from 42 to 64 years, mean \pm SD (52.4 \pm 11.6 years). The fourth group (control) contains 50 persons (66% males and 34% females), their ages ranged from 38 to 63 years, mean \pm SD (50.6 \pm 12.7 years). It should be noted that, there were significant differences in the ages and the genders among the studied groups (Table 1).

Serological and Biochemical Parameters

For serological markers, group I patients were positive for HBsAg (100%), HBeAg (55%), HBcAg (77.5%) and Anti-HBe (75%). Group II patients were positive for HBsAg (100%), HBeAg (60%), HBcAg (86.66%) and Anti-HBe (73.33%). Group III patients were positive for HBsAg (100%), HBeAg (46.66%), HBcAg (80%) and Anti-HBe (60%). Control group persons were negative with (100%) for HBsAg, HBeAg, HBcAg and Anti-HBe-IgM, but were positive for Anti-HBe (24%) and Anti-HBs (92%). P values were \leq 0.05 and significant (Fig. 1).

For Biochemical parameters, the total protein levels varied with a mean \pm SD (4.22 \pm 1.15), (3.12 \pm 1.6), (3.33 \pm 2.40) and (6.65 \pm 2.11) in group I, group II, group III and control group, respectively. The serum albumin levels varied with a mean \pm SD (2.01 \pm 1.12), (2.18 \pm 0.6), (1.16 \pm 0.9) and (3.12 \pm 1.16) in group I, group II, group III and control group, respectively. The AST activities varied with a mean \pm SD (19.8 \pm 11.4), (28.4 \pm 16.1), (26.3 \pm 6.1) and (7.3 \pm 6.4) in group I, group II, group III and control group, respectively. The ALT activities varied with a mean \pm SD (22.3 \pm 10.4), (33.6 \pm 6.8), (26.9 \pm 10.5) and (9.12 \pm 4.15) in group I, group II, group III and control group, respectively. The GGT activities varied with a mean \pm SD (52.4 \pm 11.7), (58.6 \pm 10.8), (51.3 \pm 6.7) and (18.3 \pm 6.1) in group I, group II, group III and control group, respectively. The total bilirubin levels varied with a mean \pm SD (2.6 \pm 1.33), (2.91 \pm 0.51), (2.41 \pm 0.9) and (0.61 \pm 0.41) in group I, group II, group III and control group, respectively. Finally, the AFP levels varied with a mean \pm SD (22.4 \pm 10.1), (65.4 \pm 12.8), (216 \pm 24.1) and (7.13 \pm 2.1) in group I, group II, group III and control group, respectively. P values were \leq 0.05 and were significant (Table 2).

HBV DNA Quantification

HBV DNA quantification titers (IU/ml) were increased in group I with a mean \pm SD (24.3 \times 104 \pm 6.8 \times 102), more than groups II and III with a mean \pm SD (12.8 \times 104 \pm 4.3 \times 102) and (64.8 \times 103 \pm 18.7 \times 102), respectively. P value was \leq 0.05 and significant (Table 3).

Gene Expression of *c-myc* and *p53*

For the *c-myc* gene expression level (2^{-ddct}), the results showed that significant increasing in different studied groups (chronic, cirrhosis and HCC) compared to control group (1.38, 1.47, 5.59 and 1.00, respectively). On the contrary, *p53* gene expression level was significantly decreased in the same groups (chronic, cirrhosis and HCC) compared to control group (0.82, 0.65, 0.33 and 1.00, respectively). P values were \leq 0.05 is significant (Table 4).

Distribution of *c-myc* and *p53* Genotypes

In the four studied groups, PCR products for *c-myc* gene (intron 8) were digested with *HhaI* restriction enzyme and generated three polymorphic alleles: Homozygous cystine genotype (CC = 681 bp), heterozygous glycine cystine genotype (CG = 681, 480 and 201 bp) and homozygous glycine genotype (GG = 480 and 201 bp) as shown in Fig. 2A and 2B. The frequencies of *c-myc* genotypes showed that CC genotype was predominant in HCC group (90%) more than cirrhosis, chronic and control groups (73.33, 22.5 and 6%, respectively). GG genotype was predominant in control group (70%) more than chronic, cirrhosis and HCC groups (67.5, 6.66 and 6.66%, respectively). While, GC genotype was high in control group (24%) more than cirrhosis, chronic and HCC groups (20, 10 and 3.33%, respectively) as shown in Fig. 4.

For *p53* gene (codon 72), PCR products were digested with *Bsh1236I* restriction enzyme and yielded three polymorphic alleles: Homozygous proline genotype (PP = 296 bp), heterozygous proline arginine genotype (PA = 296, 169 and 127 bp) and homozygous arginine genotype (AA = 169 and 127 bp) as can be seen in Fig. 3A and 3B. The frequencies of *p53* genotypes revealed that PP genotype was predominant in control group (88%) more than chronic, cirrhosis and HCC groups (30, 30 and 6.66%, respectively). AA genotype was predominant in HCC group (73.33%) more than chronic, cirrhosis and control groups (50, 10 and 4%, respectively). While, PA genotype was predominant in cirrhosis group (60%) more than chronic, HCC and control groups (20, 20 and 8%, respectively) as shown in Fig. 5. The results indicated that there are significant differences in *c-myc* and *p53* genotype distributions in the four studied groups and P values \leq 0.05.

Table 1: Demographic data of the four groups: Chronic, Cirrhosis, HCC and control

Groups	Case number	Age (years) (mean ± SD)	Sex (n, %)	
			Male	Female
Group I (Chronic HBV)	40	39.6±12.3	28 (70)	12 (30)
Group II (Cirrhosis-HBV)	30	42.3±13.8	21 (70)	9 (30)
Group III (HCC-HBV)	30	52.4±11.6	24 (80)	6 (20)
Control	50	50.6±12.7	33 (66)	17 (34)

SD is Standard deviation and HCC is Hepatocellular carcinoma

Table 2: Biochemical parameters of patients' groups (I-III) and control

Parameters	Group I (Chronic) (mean ± SD)	Group II (Cirrhosis) (mean ± SD)	Group III (HCC) (mean ± SD)	Control (mean ± SD)	P. values
TP (g/dl)	4.22±1.15	3.12±1.6	3.33±2.40	6.65±2.11	0.032
ALB (g/dl)	2.01±1.12	2.18±0.6	1.16±0.9	3.12±1.16	0.044
AST (U/l)	19.8±11.4	28.4±16.1	26.3±6.1	7.3±6.4	0.020
ALT (U/l)	22.3±10.4	33.6±6.8	26.9±10.5	9.12±4.15	0.010
TB (mg/dl)	2.6±1.33	2.91±0.51	2.41±0.9	0.61±0.41	0.040
GGT(U/l)	52.4±11.7	58.6±10.8	51.3±6.7	18.3±6.1	0.001
AFP (ng/ml)	22.4±10.1	65.4±12.8	216±24.1	7.13±2.1	0.077

P value ≤0.05 is significant, TP is total protein, ALB is albumin, AST is aspartate aminotransferase, ALT is Alanine Aminotransferase, TB is Total Bilirubin, GGT is Gamma Glutamyl Transpeptidase, AFP is Alfa Feto-Protein and U/L is Unit per liter

Table 3: HBV DNA titers in chronic, cirrhosis and HCC groups

Groups	HBV DNA titers (IU/ml), (mean ± SD)	P. value
Group I(Chronic)	24.3×104±6.8×10 ²	0.029
Group II(Cirrhosis)	12.8×104±4.3×10 ²	
Group III(HCC)	64.8×103±18.7×10 ²	

P value ≤0.05 is significant and IU/ml is international unit per milliliter

Table 4: Genetic expression of c-myc and p53 genes in patient groups (I-III) and control

Groups	c-myc gene expression (2 ^{-ddct})	p53 gene expression (2 ^{-ddct})	P. value
Group I(Chronic)	1.38	0.82	0.001
Group II (Cirrhosis)	1.47	0.65	
Group III (HCC)	5.59	0.33	
Control	1.00	1.00	

P value ≤ 0.05 is significant

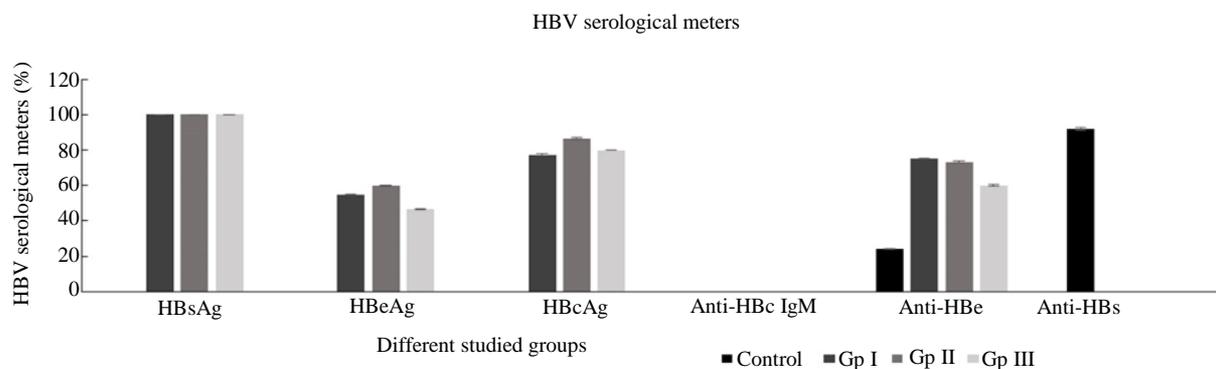


Fig. 1: Serological parameters in patient groups (I-III) and control

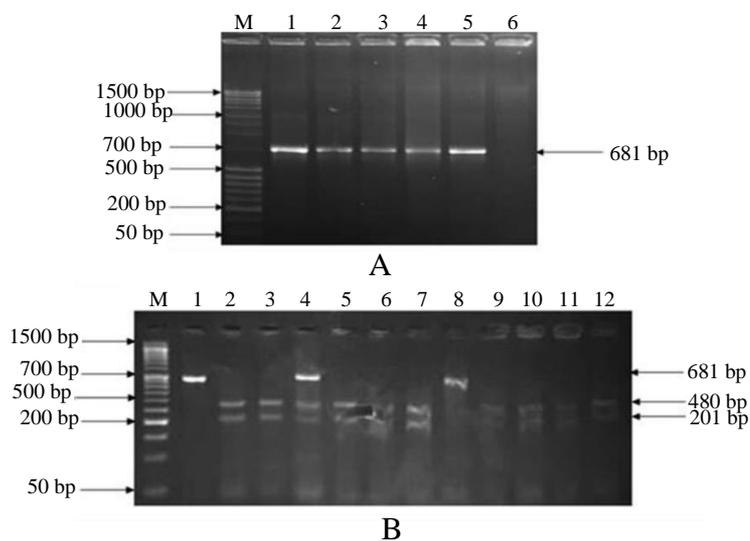


Fig. 2: A: Electrophoresis gel of c-myc PCR products. Lane M = DNA ladder (1.5 Kb), lanes 1-5 are c-myc PCR products (681 bp) and lane 6 is negative sample. B: Agarose gel electrophoresis of digested c-myc PCR products with HhaI restriction enzyme. Lane M is DNA ladder (1.5 kb), lane 1 is uncut band (681 bp), lanes 2, 3, 5, 6, 7, 9, 10, 11 and 12 are GG genotype (480 and 201 bp), lane 4 is CG genotype (681, 480 and 201 bp) and lane 8 is CC genotype (681 bp)

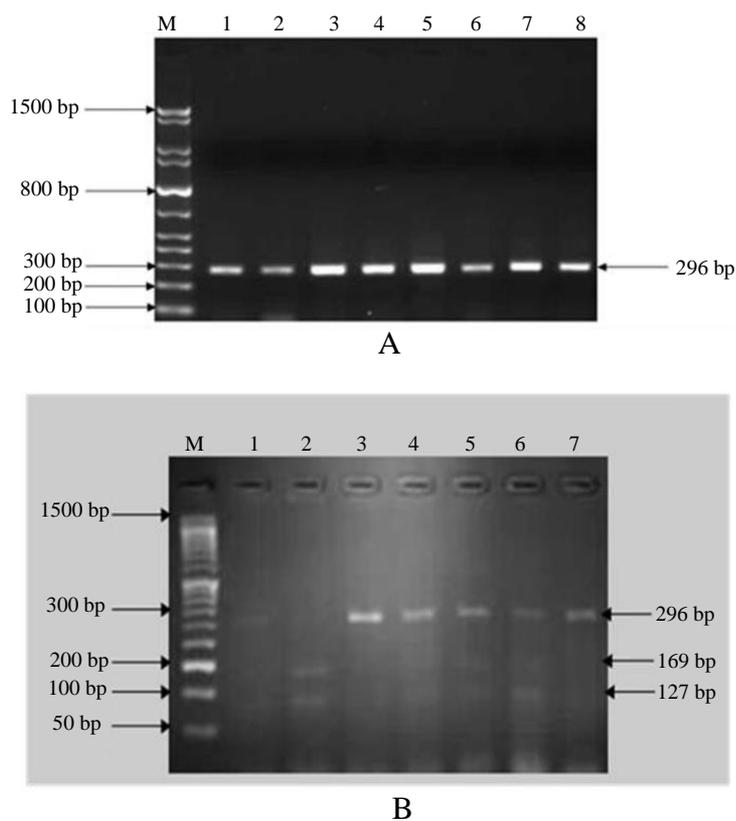


Fig. 3: A: Electrophoresis gel of p53 PCR products. Lane M: DNA ladder (1.5 K b), lanes 1 to 8 are p53 PCR products (296 bp). B: Agarose gel electrophoresis of digested p53 PCR with Bsh1236I restriction enzyme. Lane M is DNA ladder (1.5 kb), lane 1 is uncut band (296 bp), lane 2 is AA genotype (169 and 127 bp), lanes 3 and 7 are PP genotype (296 bp) and lanes 4, 5 and 6 are PA genotype (296, 169 and 127 bp)

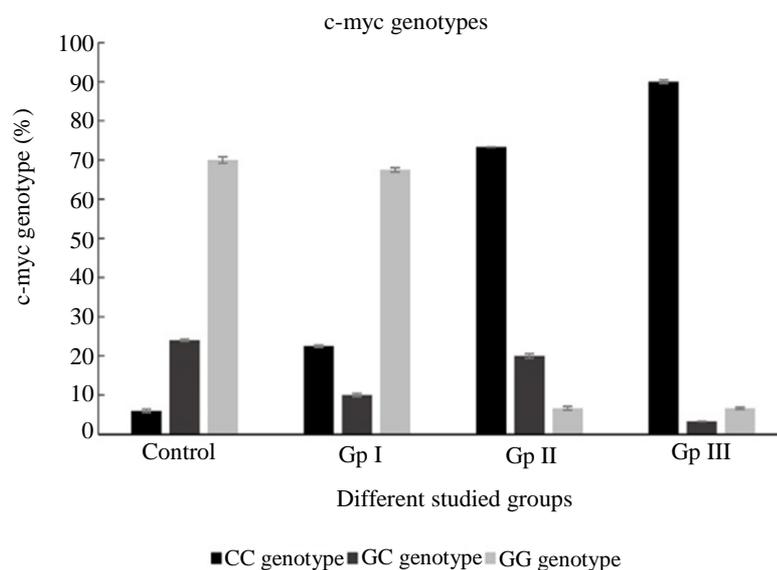


Fig. 4: Frequencies of c-myc genotypes in patient groups (I-III) and control

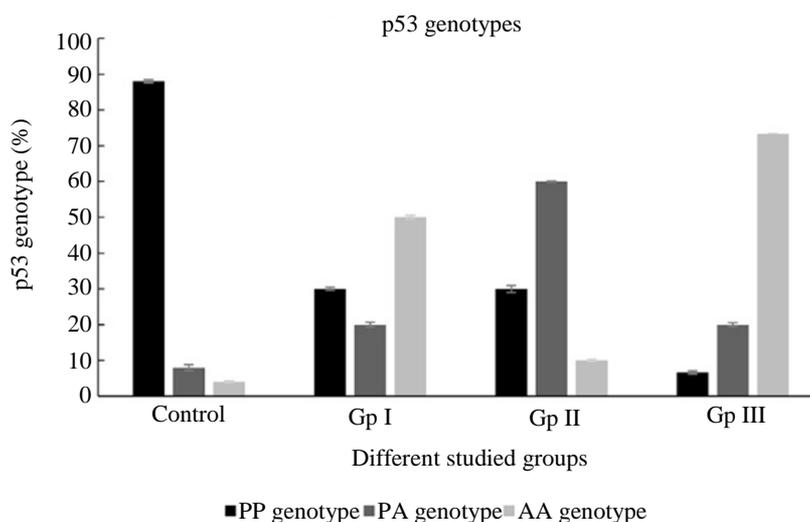


Fig. 5: Frequencies of p53 genotypes in patient groups (I-III) and control

Discussion

Chronic viral hepatitis, such as HCV and HBV were an important global health problem, as well as, the major etiology of End-Stage Liver Disease (ESLD), such as cirrhosis and HCC worldwide (Kao and Chen, 2005). Several factors are known for affecting the clinical outcomes of chronic viral hepatitis, inclusive viral, host, environmental and genetic factors (Lok and McMahon, 2001). Hepatocarcinogenesis is a complex and multistep process which influenced by both oncogenes and tumor suppressor genes (Bruix and Sherman, 2005). In this study, we detected the vital impact of c-myc and p53

genetic expression and polymorphism on the progression of HBV infection in three different stages (chronic, cirrhosis and HCC) among Egyptian population. Biochemical, serological and molecular parameters of these patients and control were determined.

In the current study, the results showed significant increasing enzyme activity of AST, ALT and GGT, in addition to, TB and AFP levels ($P = 0.02$, $P = 0.01$, $P = 0.001$, $P = 0.04$ and 0.077 , respectively) in chronic, cirrhosis and HCC groups compared with the control group. While, the results showed significant decreasing of serum TP and ALB levels ($P = 0.032$ and $P = 0.044$, respectively). However, the results also showed that a

reduction in the levels of both TP and ALB. While, a rising in serum TB, ALT, AST, GGT and AFP levels in all studied groups compared to the control group. This is because of the increasing in progression of HBV infection, incessant hepatocytes inflammation and injury, leading to hepatocytes laceration and infiltration of their contents. These results agree with our related previous study (Koutb *et al.*, 2017) which revealed that lipid peroxidation and enzyme activity of AST, ALT and GGT, in addition to TB level were increased in acute, chronic, cirrhosis and HCC groups compared to control group (Kaneko *et al.*, 1989).

For determination of HBV infection, testing for the presence of HBsAg should be the initial diagnostic test, because the Anti-HBc was not used as a screening examination to determine previous exposure to the HBV. Where, Anti-HBc was the first antibody which produced after HBV infection, additionally, the only discovered marker in the window period. However, isolated anti-HBc refers to the presence of anti-HBc in the serum without HBsAg or HBsAb and that is because of the resolved HBV infection, due to that HBsAb had declined to the undetectable level. HBV testing during the window period or chronic infection, HBsAg cannot be detected due to protein mutation which makes it undetectable by certain diagnostic assays (Akbaş *et al.*, 2012). In this study, our results indicated that the HBV serological markers, such as HBeAg, HBcAg and Anti-HBe were significantly decreased in HCC and cirrhosis groups compared to chronic group ($P = 0.022$, $P = 0.001$ and $P = 0.041$, respectively). This study also showed that significant decreasing in HBV DNA titres (Viremia Level (VL)) in HCC and cirrhosis groups compared to chronic group ($P = 0.048$, $P = 0.037$ and $P = 0.029$, respectively). These results agree with previous studies reporting that the patients with less liver damage had higher viral load (Oh *et al.*, 2015; Kao, 2003).

C-myc gene expression in the HL-7702 cell line was significantly increased after transient or stable transfection with HBV X gene expression vector. C-myc protein can cause cells to apoptotic killing in definite conditions, such as, exposure to Tumour Necrosis Factor alpha (TNF α) or to another factors (Nishida and Goel, 2011). So, c-myc may do as an significant medium in HBx/mediated apoptosis. However, c-myc gene expression level was observed high with 74% in HCC tissues, in addition, an over expression of c-myc gene was involved in both the liver regeneration and the hepatocarcinogenesis (Koutb *et al.*, 2017). Moreover, it is also a significant indicator of both the malignant potential and the poor prognosis (Almeida Neto *et al.*, 2001). Our results showed that significant increasing in different studied groups (Chronic, Cirrhosis and HCC) compared to control group (1.38, 1.47, 5.59 and 1.00, respectively). All P values were ≤ 0.05 and significant.

These results agree with our previous study (Koutb *et al.*, 2017) which showed significant increasing of c-myc gene expression in different studied groups (chronic, cirrhosis and HCC) related with HCV infection compared to control group (1.17, 1.82, 3.33 and 0.32, respectively) (Kaneko *et al.*, 1989).

In several studies, the role of pivotal c-myc gene was demonstrated in diverse cancers (Nilsson and Cleveland, 2003). Association of c-myc gene expression together with the liver carcinogenesis was the first identification through the high expression of c-myc gene in both chronic liver disease and HCC, in addition, the frequent c-myc amplification in the liver cancer tissues, which was ordinarily seen in patients at the younger ages with poor prognosis (Chu and Lok, 2002). Moreover, HBV chronic infection was often associated with HCC in Asian countries and HBx gene has been implicated in HBV mediated HCC (Heo *et al.*, 2003). In this study, frequencies of c-myc genotypes in the four studied groups showed that CC genotype was predominant in HCC group (90%) more than cirrhosis, chronic and control groups (73.33, 22.5 and 6%, respectively). GG genotype was predominant in control group (70%) more than chronic, cirrhosis and HCC groups (67.5, 6.66 and 6.66%, respectively). GC genotype was high in control group (24%) more than cirrhosis, chronic and HCC groups (20, 10 and 3.33%, respectively).

Direct association between HBX and p53 proteins is observed with HBX-GST fusion proteins in vitro or in vitro translated proteins (Su *et al.*, 2001). HBX gene directly interacts with transcription machinery such as RNA polymerase, TATA- binding protein, TF/IIB and other cellular proteins like p53 (a tumor suppressor protein). Subsequently, the pleiotropic activity of HBX gene may contribute to the modulation of gene expression, which finally leads to the formation of liver cancer (Su *et al.*, 2001). As mentioned in the previous studies, Hepatitis B virus X (HBx) protein can bind to the C terminus of p53 and then induces its sequestration from the nucleus to the cytoplasm, hence, inhibition of its effect on both cell cycle arresting and DNA repairing. In the present study, p53 gene expression was decreased significantly in different studied groups (chronic, cirrhosis and HCC) compared to control group (0.82, 0.65, 0.33 and 1.00, respectively). These results agree with our previous study that showed that p53 gene expression was decreased significantly in the different studied groups (chronic, cirrhosis and HCC) related to HCV infection compared to control group (4375, 3842, 525 and 5498, respectively) (Kaneko *et al.*, 1989).

Loss of p53 function due to genomic alteration or interaction with viral agents has postulated as a critical step in the development of HCC (Sheen *et al.*, 2003). Yuen *et al.* (2001) reported that the p53 gene function loss due to the genomic alteration or the interaction with

viral agents has presumed as a critical step in the development of Hepatocellular Carcinoma (HCC). Some previous studies (Poeta *et al.*, 2007; Dumont *et al.*, 2003; Cheng, 2002) have mentioned that the P53 gene has ten polymorphisms in the human genome and the polymorphisms of both exon 4 (codon 72) and exon 7 (codon 249) were the most associated to the development of Hepatocellular Carcinoma (HCC). Additionally, Previous study has reported that that the Arg variant is a less efficient DNA repair molecule and therefore Arg/Arg carriers may have an increased frequency of chromosomal aberrations and a high level of genomic instability (Chan *et al.*, 2004). The recent studies had suggested that the Pro allele at the codon 72 of p53 gene might have a synergistic effect on HCC development for subjects with chronic HBV disease and family history of HCC in first-degree relatives (Yuen *et al.*, 2003). In our study, the results showed that p53 genotypes frequencies were as follows: PP genotype was predominant in control group (86.66%) more than chronic, cirrhosis and HCC groups (15, 10 and 20%, respectively). AA genotype was varied in control, chronic and HCC groups (13.33, 25 and 30%, respectively) and absent in cirrhosis group. PA genotype was predominant in cirrhosis group (90%) more than chronic and HCC groups (60 and 50%, respectively) and absent in control group.

Conclusion

In Conclusion, p53 (codon 72) and c-myc (intron 8) gene expression and polymorphism have the risk effect on the progression of HBV infection. Consequently, the interaction of both p53 and c-myc genes play an important role in the etiology of Hepatocellular Carcinoma (HCC), which correlated to HBV. However, more studies on human populations, with well-designed and larger samples, are needed to validate our finding. Finally, the application of p53 codon 72 and c-myc gene expression and polymorphism could be used as genetic markers for prediction, monitoring and treatment of different stages of HBV infection.

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Author Contributions

Fayed Koutb: Participated in all experiments, coordinated the data-analysis and contributed to the writing of the manuscript.

Salah Abdel-Rahman: Designed the research plan, organized the study analysis and contributed to the writing of the manuscript.

Ehab Hassouna: Coordinated the samples collection and clinical investigation.

Amany Haggag: Participated in the experiments, coordinated the data-analysis.

Ethics

The study was conducted in accordance with the Declaration of Helsinki and the protocol was approved by the Ethics Committee of the Faculty of Medicine, Alexandria University.

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