American Journal of Cancer Prevention, 2015, Vol. 3, No. 2, 27-34
Available online at http://pubs.sciepub.com/ajcp/3/2/2

DOI:10.12691/ajcp-3-2-2

Detection of BCL2 Polymorphism in Patient with Hepatocellular Carcinoma

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Received March 26, 2015; Revised April 05, 2015; Accepted April 10, 2015

Abstract **Introduction:** Despite advances in the knowledge of the molecular virology of hepatitis C virus (HCV), the mechanisms of hepatocellular injury in HCV infection are not completely understood. Hepatitis C viral infection (HCV) influences the susceptibility to apoptosis. This could lead to insufficient antiviral immune response and persistent viral infection. **Aim of this study**: Is to examine whether BCL-2 gene polymorphism at codon 43 (+127G/A or Ala43Thr) has an impact on development of hepatocellular carcinoma caused by chronic hepatitis C infection among Egyptian patients. **Subjects and Methods:** The study included three groups; group 1: composed of 30 patients with hepatocellular carcinoma (HCC), group 2 composed of 30 patients with chronic hepatitis C infection (CHC) and group 3 composed of 30 healthy subjects matching the same age and socioeconomic status were taken as a control group. Gene polymorphism of BCL2 (Ala43Thr) was evaluated by Restriction fragment length polymorphism (PCR-RFLP) techniqueand measured for all patients and controls. **Results:** The summed 43Thr genotype was more frequent and statistically significant in HCC patients as compared to control group. This genotype of BCL2 gene may inhibit the programmed cell death which leads to disturbance in tissue and cells homeostasis and reduction in immune regulation. This result leads to viral replication and HCV persistence. Moreover, virus produces variety of mechanisms to block genes participated in apoptosis. This mechanism proves that CHC patients who have 43Thr genotype are more susceptible to HCC. Correlation coefficients between AFP versus ALT and AST were statistically significant. **Conclusion:** The data investigated for the first time that BCL2 polymorphism is associated with the susceptibility to HCC in Egyptian populations and might be used as molecular markers for evaluating HCC risk. This study clearly demonstrated that CHCexhibit a deregulation of apoptosis with the disease progression. This provides an insight into the pathogenesis of chronic hepatitis C infection, and may contribute to the therapy.

Keywords: *BCL2 gene, hepatitis c virus, hepatocellular carcinoma, sensitivity, specificity, apoptosis*

Cite This Article: Mohamed Abdel-Hamid, Olfat Gamil Shaker, Doha El-Sayed Ellakwa, and Eman Fathy Abdel-Maksoud, "Detection of BCL2 Polymorphism in Patient with Hepatocellular Carcinoma." *American Journal of Cancer Prevention*, vol. 3, no. 2 (2015): 27-34. doi: 10.12691/ajcp-3-2-2.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors in the world. Currently, surgical resection, liver transplantation, and local ablation are considered curative therapeutic practices for HCC. The diagnosis of HCC without pathologic confirmation is achieved by analyzing serum alpha-fetoprotein (AFP) levels combined with imaging techniques, including ultrasonography, magnetic resonance imaging, and computerized tomography,although progress has been made in the diagnosis and management of HCC, its prognosis remains dismal. AFP is the biomarker most widely used to test for HCC, but the sensitivity and specificity of AFP vary widely, and total AFP is not always specific, especially when HCC is in its early stages [17].

Hepatitis C virus (HCV) is often established as a persistent infection to cause chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma which is a significant health problem around the world [7]. Egypt has the highest HCV prevalence in the world. It estimated HCV prevalence among the 15 – 59 years age group to be 14.7% [14]. Egypt has the highest prevalence of genotype 4, which is responsible for more than 90% of infections and is considered a major cause of chronic hepatitis, liver cirrhosis, hepatocellular carcinoma, and liver transplantation in the country, despite advances in the knowledge of the molecular virology of hepatitis C virus (HCV), the mechanisms of hepatocellular injury in HCV infection are not completely understood [20]. After infection is established, multiple factors influence the host–virus interaction, resulting in a unique individual disease pattern. The available reports are in favor of a destructive mechanism mediated by the host immune system rather

than a direct cytopathic effect of the virus,this comprises both a nonspecific immune response and HCV specific humoral and cellular immune response [19]. Apoptosis is a physiological form of cell death evolved in multicellular organisms to eliminate unwanted cells through a coordinated series of enzymatic steps and controlled by inhibitors at each step. The B cell lymphoma leukemia 2 (Bcl-2)proto-oncogene is an established survival factor whose physiological function is to prevent apoptosis, and the activity of Bcl-2 may be counteracted by dimerization with bax [12]. Bax is proapoptotic and, together with other death agonist members, increases sensitivity to death-inducing signals. A delicate balance normally exists in the body between the anti-apoptotic and pro-apoptotic regulators of apoptosis to ensure the proper survival and turnover of different body cells butimbalance in the apoptotic pathway occurs in disease scenarios [6]. The existence of the Bcl-2 (B-cell lymphoma-2) gene was reported nearly 30 years ago. Yet, Bcl-2 family group of proteins still surprises us with their structural and functional diversity. Since the discovery of the Bcl-2 family of proteins as one of the main apoptosis judges, the precise mechanism of their action remains a hot topic of intensive scientific research and debates [12]. An in vitro study of Ala43Thr (G128A) polymorphism in BCL2 anti-apoptotic gene showed that Ala43Thr genotype increases the survival of cells, while the threonine variant acts as a suppressed haplotype for the anti-apoptotic factor [11]. To the best of our knowledge, to date, there is no reported study of BCL2 Ala43Thr polymorphism in HCC patients.

2. Subjects and Method

2.1. Study Subjects

Ninety individuals were included in this study and divided into three groups; sixty outpatients with chronic liver disease classified into two groups; group I:include 30 patients with Hepatocellular carcinoma (HCC), group II:include 30 patients of chronic hepatitis C and group III: include thirty healthy controls. Patients visiting outpatient clinics of National Hepatology and Tropical Medicine Research Institute Clinics, Kasr El-Aini Hospital, Cairo University, Egypt and diagnosed as chronically infected HCV and HCC. The study protocol and informed consent were approved by the Ethics Committee of National Cancer Research Institute, Cairo University and National Hepatology and Tropical Medicine Research Institute Clinics.

2.2. Patients inclusion criteria

Patients that participated in the study fulfilled the inclusion criteria included: age 48–65 years, elevated ALT and AST (N37 IU/L); within 6 months prior to entry the study, positive HCV antibodies, detectable HCV-RNA, HCV genotype 4, liver biopsy showing histological evidence of chronic hepatitis or/withhepatocellular carcinoma.

2.3. Patients Exclusion Criteria

Hepatitis B surface antigen (HBsAg) seropositivity, autoimmune hepatitis, co- infection with the human immunodeficiency virus (HIV) and the presence of any chronic systemic illness were excluded from this study.

2.4. Biochemical Tests

All patients were subjected to the following: Full clinical examination and biochemical tests which included: a) The HCV antibody was determined with the HCV enzyme immunoassay (EIA) version 3 (Ortho Diagnostics). Colorimetric determination of Alanine aminotransferase (ALT) activity was done according to the Reitman and Frankel (1975) using kits provided by dp International. Colorimetric determination of Aspartate aminotransferase (AST) was done using kits provided by UniLab Biotechnology. AFP was done according to the "DS-EIA-AFP" is a one-step immunoassay, based on principle of sandwich method.

2.5. Genotyping of BCL-2 (Ala43Thr) SNP

The polymorphism of BCL2 gene at point (Ala43Thr) was studied in the current study to test whether BCL2 gene polymorphism has an impact on development of HCC using Restriction fragment length polymorphism (PCR-RFLP) technique.

2.5.1. DNA Extraction

The DNA was extracted from Buffy coat of all patients and healthy controls using QIAamp DNA blood Mini kit. The extraction kit was supplied by (Qiagen Inc., Valencia, USA).

2.5.2. Polymerase Chain Reaction

Nested PCR was done using the thermal cycler (Biometra®, Gottingen, Germany) for amplifying the BCL-2 gene. PCR reagents weresupplied by Promega,Inc., UK. Generalreactionsprimers wereas follows; all primer sequences were from (5′ to 3′), the forward primer of PCR1 (F1); TGG GGT GGG AGC TGG GGC GAG AG, the reversed primer of PCR1 (R1); GGT CAG CAG CGG CGA GGT CC, which yields a (281 bp) product. The nested forward primer of PCR2 (F2): CCC GTT GCT TTT CCT CTG GGA. The nested reversed primer of PCR2 (R2): GGG AGG AGA AGA TGC CCG CCG CGG GG, resulting in a (178 bp) amplicon. The PCR1 reaction conditions were as follows; denaturation at 95 °C for 30 s, annealing at 63 °C for 30 s, and extension at 72 °C for 30 s. The PCR2 reaction conditions were as follows: denaturation at 95 °C for 30 s, annealing at 62 °C for 30 s, and extension at 72 °C for 30 s. Amplified DNA plus a 6× DNA loading buffer were loaded and electrophoresed on a 2% agarose gel prepared in 1× TBE buffer and containing ethidium bromide (0.5 μg/ml) for 40 min at 100 Volts (V). Gene Ruler 100 bp DNA Ladder (Fermentas, Life Sciences) was routinely used as a molecular weight standard as a reference in the agarose gel. The gel was visualised on a UV gel documentation computerized system. Images were manipulated by BioDocAnalyze software program (Biometra®, Goettingen, Germany).

2.5.3. Restriction Fragment Length Polymorphism (RFLP) Technique

The 2nd PCR product (178 bp amplicon) was incubated at 37 °C overnight with BglI restriction enzyme using (PCR-RFLP) technique to identify the three different types of BCL-2. Complete digestion of the wild type

homozygote, gives; the Ala 43 genotype (157 bp+21 bp), mutant type homozygote, gives; the Thr 43 genotype undigested (178 bp) and heterozygote, gives; the Ala 43 Thr genotype (178 bp+157 bp+21 bp). BglI was obtained from (Fermentas, Life Sciences). Digested PCR product was electrophoresed on a 2% agarose gel. Gene Ruler 50 bp DNA Ladder (Fermentas, Life Sciences) was used as a molecular weight standard as a reference in the agarose gel. The gel was visualized on a UV gel documentation computerized system. Images were manipulated by BioDocAnalyze software program (Biometra®, Goettingen, Germany).

2.6. Statistical Analysis

Data were statistically described in terms of mean, standard deviation (±S.D.), frequencies (number of cases) and relative frequencies (percentages) when appropriate. Comparison of variables between the study groups was done using (ANOVA) test. For comparing categorical data, Chi square (χ^2) test was performed. A probability value (*P* value) less than 0.05 was considered statistically significant. Statistical calculations were done using statistical computer program: SPSS (Statistical Package for the Social Science; SPSS Inc., Chicago, IL, USA, 2001). Receiver-operating characteristics curves (ROC) were constructed to evaluate the diagnostic performance of the serum markers in discriminating HCC from other groups. Sensitivity, specificity, and diagnostic accuracy were calculated in accordance with standard methods. P<0.05 for a two-tailed test was considered statistically significant.

3. Results

3.1. Patients Clinical Features

The clinical featuresand laboratory data of sixty patients infected with chronic HCC and CHCand the thirty healthy control individuals are summarized in (Table 1). ALT, AST and AFP were significantly associated with HCC and CHC infected patients compared to healthy control individuals (P<0.05).

Table 1. Comparison between statistics of the laboratory data of the Hepatocellular carcinoma group,Chronic hepatitis C group and the control group

Variables	HCC group(group I)	CHC(group II)	Control group(group III)	F-value	p-value
	Mean ± SD	Mean ± SD	Mean ± SD		
AST (IU/L)	169.13 ± 177.64	40.77 ± 33.64	29.60 ± 9.50	16.507	0.000***
ALT (IU/L)	169.73 ± 173.32	38.30 ± 32.06	26.60 ± 9.50	18.244	0.000***
AFP (IU/ml)	344.5 ± 473.68	14.26 ± 39.85	3.402 ± 2.76	14.971	0.000***

AST: Aspartate aminotransferase
ALT: Alanine aminotransferase
AFP: Alpha Fetoprotein
p –Value > 0.05 is non- significant.
*p < 0.05
**p <0.01
***p <0.001
NS = Not Significant
Different symbol indicate significance.

The mean level of (AST and ALT) in the HCC (169.13 ± 177.64, 169.73 ± 173.32) group were significantly higher than the mean of (AST and ALT) in the control group (29.60 ± 9.50 IU/L, 26.60 ± 9.50) at P< 0.001 (Table 1). The mean level of (AST and ALT) of the CHCgroup (40.77 ± 33.64 IU/L, 38.30 ± 32.06 IU/L) were significantly higher than in the control group at P< 0.001 (Table 1).

Table 2. Correlation between AFP and other laboratory data in all studied groups

Correlation of AFP with	HCC gp I		CHC gp II		Control gp	
	R	p-value	R	p-value	R	p-value
AST (IU/L)	0.608**	P < 0.001	0.393*	P < 0.05	-0.187	P> 0.05
ALT (IU/L)	0.590**	P < 0.01	0.378*	P < 0.05	-0.187	P> 0.05

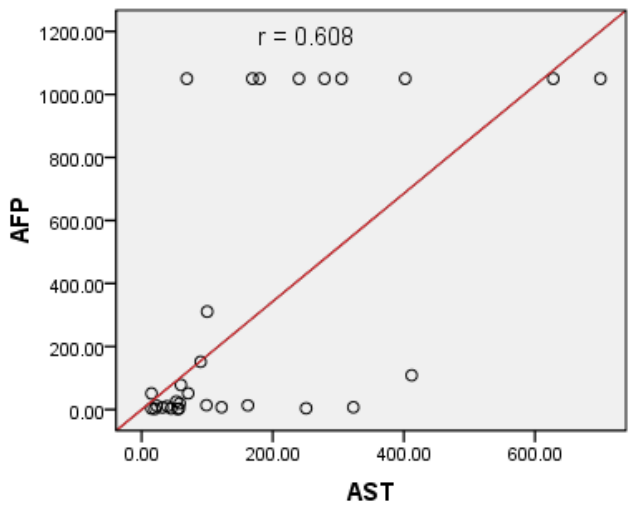

Figure 1. Correlation between AFP and AST in the HCC group

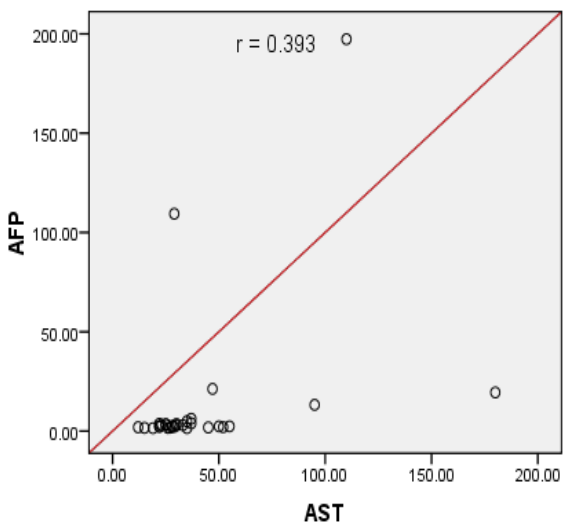

Figure 2. Correlation between AFP and AST in the CHC group

Also, the mean level of AFP in the HCC group (344.5 ± 473.68 IU/ml) was significantly higher than the mean of AFP in the control group (3.402 ± 2.76 IU/ml) at P< 0.001 (Table 1). The mean level of AFPof the CHCgroup (14.26 ± 39.85 IU/ml) was significantly higher than in the control group at P< 0.001 (Table 1).

In Table 2 Showed the correlation between AFP and other laboratory data of the HCC group and CHC group, showing a significant positive correlation between AFP and each of AST (r = 0.608) (r = 0.590) at P< 0.01, ALT (r = 0.393) (r = 0.378) at P< 0.05 in HCC groups and CHCgroups respectively which represented in Figure 1, Figure 2, Figure 3 and Figure 4 respectively.

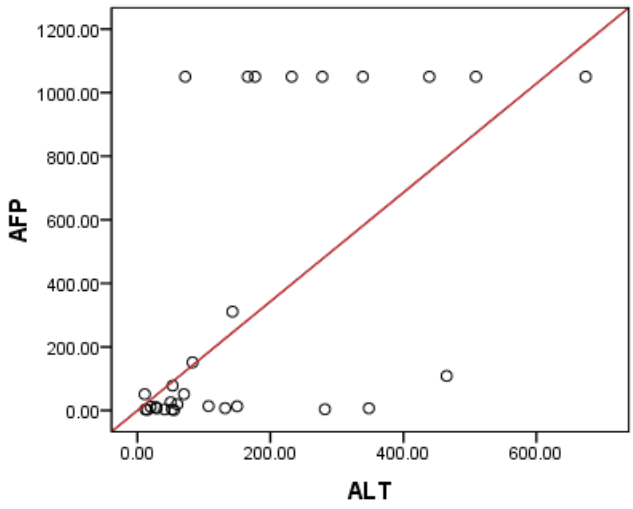

Figure 3. Correlation between AFP and ALT in the HCC group

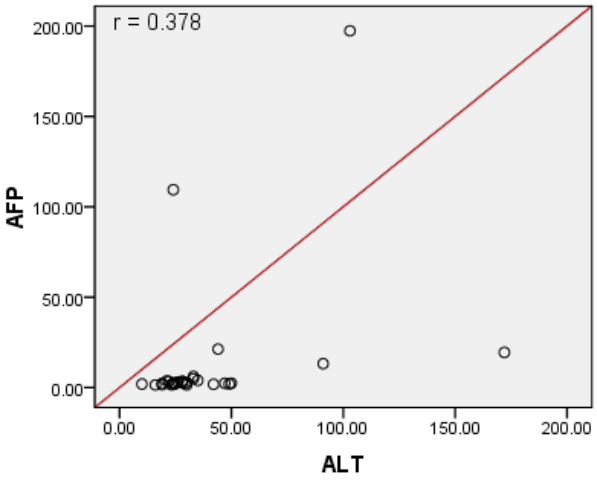

Figure 4. Correlation between AFP and ALT in the CHC group

When HCC patients were compared to CHC patients and control group, AUC for AFP. The best cutoff value for diagnosis of HCC using AFP is 6.46 with sensitivity= 80% and specificity=85% (Figure 5).

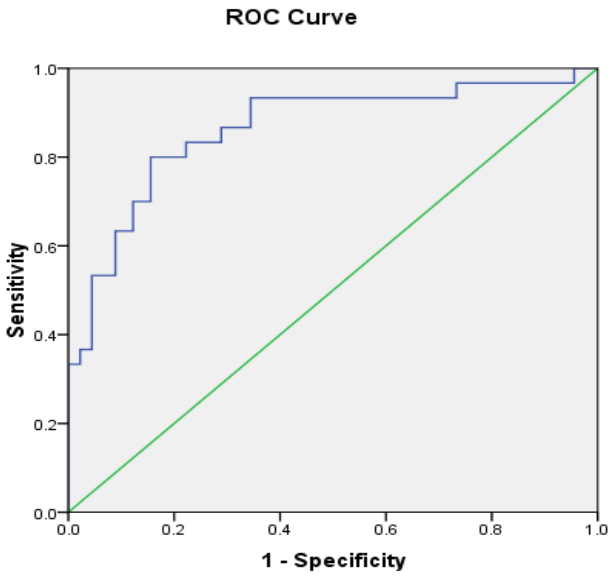

Figure 5. Receiver operating characteristic (ROC) plots curve for AFP in diagnosis of early stages of HCC versus CHC patients and healthy control

Mean of area under curve for AFP marker was 0.863.

3.2. Genotyping of BCL-2

3.2.1. Detection of BCL-2 genotypes Complete digestion of the wild type homozygote, gives; the Ala 43 genotype (157 bp+21 bp), mutant type homozygote, gives; the Thr 43 genotype undigested (178 bp) and heterozygote, gives; the Ala 43 Thr genotype (178 bp+157 bp+21 bp) (Figure 6).

3.2.2. Frequencies of BCL-2 gene SNP at +127G/A : The frequencies of BCL-2 gene SNP at +127G/A in HCC and CHCpatients and in healthy control individuals are described in Tables 3,4 and 5. Table 3 shows that CHC patients showed high frequency and a non-significant difference of BCL-2 43Thr genotype and allele when compared to healthy control individuals. Whereas, the frequencies of BCL2 gene SNP at (Ala43Thr) in HCC patients are summarized in Table 4.This table showed that HCC patients have a high frequency (about double) and significant difference of BCL2 43Thr genotype and allele as compared to healthy control individuals. While, the frequencies of BCL2 gene SNP at (Ala43Thr) in HCC patients are summarized in Table 5.This table showed that HCC patients have a high frequency and non-significant difference of BCL2 43Thr genotype and allele as compared to CHC patients.

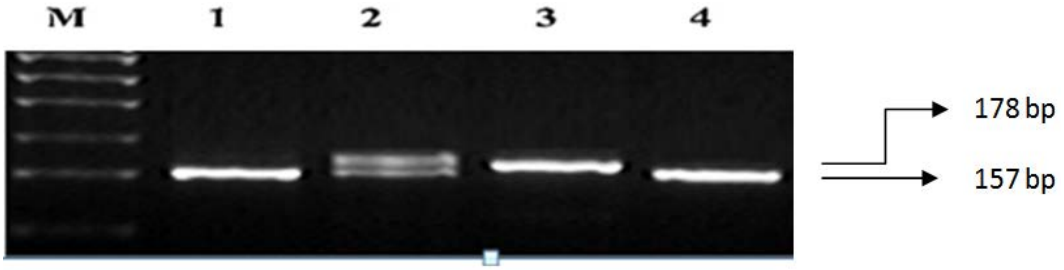

Figure 6. Agarose gel electrophoresis 2% stained with ethidium bromide showing gene polymorohism of BCL-2 after BglI digestion. BglI cuts 43Ala PCR products but does not cut 43Thr fragments.Lane M represents DNA Marker (50,150,300,450,700 and 1000 bp), lanes 1 and 4 represent the43Ala genotype (157, 21 bp), and lane 2 represent the Ala43Thr genotype (178, 157, 21 bp) lane 3 represents the 43Thr genotype (178 bp)

Table 3. The genotype and allele frequencies of BCL-2 in CHC patients as compared to healthy controls

BCL-2 genotypes and alleles	Healthy Control N=30		CHC patientsN=30		χ2 test	p-value
	n	%	n	%		
Ala 43 Ala	25	83.3 %	21	70 %	Reference	
Ala 43 Thr	5	16.7 %	7	23.3 %	0.613	0.434
Thr 43 Thr	0	0	2	6.7 %	2.268	0.224
43 Thr	5	16.7 %	9	30%	1.491	0.222
BCL-2 alleles						
43 Ala	55 (91.7%)		49 (81.7%)		Reference	
43 Thr	5 (8.3)		11 (18.3%)		2.596	0.107

Significant at $p < 0.05$
*$p < 0.05$
**$p < 0.01$
***$p < 0.001$
Not Significant (NS) at $p > 0.05$
Different symbol indicate significance.

Table 4. The genotype and allele frequencies of BCL-2 in HCC patients as compared to healthy controls

BCL-2 genotypes and alleles	Healthy Control N=30		HCC patientsN=30		χ2 test	p-value
	n	%	n	%		
Ala 43 Ala	25	83.3 %	16	53.3 %	Reference	
Ala 43 Thr	5	16.7 %	10	33.3%	3.374	0.066
Thr 43 Thr	0	0	4	13.3 %	5.488	0.033
43 Thr	5	16.7 %	14	46.6%	6.239	0.012
BCL-2 alleles						
43 Ala	55 (91.7%)		42 (70%)		Reference	
43 Thr	5 (8.3)		18 (30%)		9.09	0.003

Table 5. The genotype and allele frequencies of BCL-2 in CHC patients as compared to HCC patients.

BCL-2 genotypes and alleles	CHC patientsN=30		HCC patientsN=30		χ2 test	p-value
	n	%	n	%		
Ala 43 Ala	21	70 %	16	53.3 %	Reference	
Ala 43 Thr	7	23.3 %	10	33.3%	1.133	0.287
Thr 43 Thr	2	6.7 %	4	13.3 %	1.139	0.393
43 Thr	9	30%	14	46.6%	1.763	0.184
BCL-2 alleles						
43 Ala	49 (81.7%)		42 (70%)		Reference	
43 Thr	11 (18.3%)		18 (30%)		2.228	0.136

Table 6. The genotype and allele frequencies of BCL-2 in CHC patients as compared to healthy controls

BCL-2 genotypes and alleles	Healthy Control N=30		CHC patientsN=30		OR (95% CI)	p-value
	n	%	n	%		
Ala 43 Ala	25	83.3 %	21	70 %	1.00 (Reference)	
Ala 43 Thr	5	16.7 %	7	23.3 %	1.667 (0.461-6.030)	0.434
Thr 43 Thr	0	0	2	6.7 %	------	0.224
43 Thr	5	16.7 %	9	30%	2.143 (0.622-7.387)	0.222
BCL-2 alleles						
43 Ala	55 (91.7%)		49 (81.7%)		1.00 (Reference)	
43 Thr	5 (8.3%)		11 (18.3%)		2.469 (0.802-7.607)	0.107

Table 6 showed that the frequency of 43 Thr allele and Ala 43 Thr, Thr 43 Thr, 43 Thr (Ala 43 Thr + Thr 43 Thr) genotype showed no significant difference between CHC patients and control groups (18.3% vs 8.3%, 23.3% vs 16.7%, 6.7% vs 0%, 30% vs 16.7%).

Table 7 showed that the frequency of Thr 43 Thr was significantly higher in HCC patient than that in control group (13.3% vs 0% at $p < 0.05$). Also, the frequency of 43 Thr (Ala 43 Thr + Thr 43 Thr) genotype was significantly higher in HCC patient than that in control group (46.6% vs 16.7% at $p < 0.05$). Also, the frequency of 43 Thr allele was significantly higher in HCC patient than that in control group (30% vs 8.3% at $p < 0.05$). So, 43 Thr (Ala 43 Thr + Thr 43 Thr) genotype was associated with the risk of

HCC incidence (46.6%, 4.375 (1.32 - 14.504) p < 0.05). Also, the 43 Thr allele was associated with the risk of HCCincidence (30%, 4.714 (1.618 - 13.732) p < 0.05). While the frequency of Ala 43 Thr genotype showed no significant difference between HCC patients and control group (33.3% vs 16.7%).

Table 7. The genotype and allele frequencies of BCL-2 in HCC patients as compared to healthy controls

BCL-2 genotypes and alleles	Healthy Control N=30		HCC patientsN=30		OR (95% CI)	p-value
	n	%	n	%		
Ala 43 Ala	25	83.3 %	16	53.3 %	1.00 (Reference)	
Ala 43 Thr	5	16.7 %	10	33.3%	3.125 (0.901-10.836)	0.066
Thr 43 Thr	0	0	4	13.3 %	-----	0.033*
43 Thr	5	16.7 %	14	46.6%	4.375 (1.32-14.504)	0.012**
BCL-2 alleles						
43 Ala	55 (91.7%)		42 (70%)		1.00 (Reference)	
43 Thr	5 (8.3%)		18 (30%)		4.714 (1.618-13.732)	0.003**

Table 8. The genotype and allele frequencies of BCL-2 in CHC patients as compared to HCC patients

BCL-2 genotypes and alleles	CHC patientsN=30		HCC patientsN=30		OR (95% CI)	p-value
	n	%	n	%		
Ala 43 Ala	21	70 %	16	53.3 %	1.00 (Reference)	
Ala 43 Thr	7	23.3 %	10	33.3%	1.875 (0.585-6.008)	0.287
Thr 43 Thr	2	6.7 %	4	13.3 %	2.625 (0.426-16.164)	0.393
43 Thr	9	30%	14	46.6%	2.042 (0.707-5.895)	0.184
BCL-2 alleles						
43 Ala	49 (81.7%)		42 (70%)		1.00 (Reference)	
43 Thr	11 (18.3%)		18 (30%)		1.909 (0.811-4.493)	0.136

Table 8 showed that the frequency of 43 Thr allele and Ala 43 Thr, Thr 43 Thr, 43 Thr (Ala 43 Thr + Thr 43 Thr) genotype showed no significant difference between CHC patients and HCC patients (18.3% vs 30%, 23.3% vs 33.3%, 6.7% vs 13.3%, 30% vs 46.6%).

4. Disscusion

To the best of our knowledge, this is the first study to provide novel information of Bcl2 polymorphism effects on HCC risk in Egypt.Genetic polymorphisms may be involved in multistage of hepato-carcinogenesis, and play a role in susceptibility to the development of HCC [1]. Identification of genetic polymorphisms could clarify the pathophysiologic mechanism of carcinogenesis. Based on the genetic information, we determine the disease etiology in terms of genetic determinants to be used for identifying the high-risk individuals and perform targeting therapy to the individual's genetic make-up. In our study, we carried out a case-control study to investigate the associationof Bcl2 polymorphism and HCC risk.

The current study was conducted on 90 subjects (group 1 composed of 30 patients with HCC, group 2 composed of 30 patients with CHC, group 3 composed of 30 healthy subjects matching the same age and socioeconomic status were taken as a control group with mean ages of (55.60 ± 6.030, 56.350 ± 5.985 and 53.25 ± 6.09 years respectively).

In the present study, we analyzed several factors which are associated with CHC infection and are known to be associated with disease prognosis. These factors are gender, age, liver enzymes (ALT and AST), α-fetoprotein (AFP), and viral genotype.

The mean serum value ofAFPinEgyptian HCC patients was significantly higher than those of the CHCpatients followed by those of control group at (p<0.001) as shown in (Table 1). These results were in agreement with the studies of Taketa 1990, Niederau et al., 1998 and Marcellin 1999. Based on the ROC analysis, AFP in HCC patients relative to CHC patients and healthy control had good area under the curve (AUC), sensitivity, specificity, (0.863, 80%, 85% respectively P<0.0001 at cut off = 6.46 ng/ml) suggesting that AFP could performs as a rule-in test.

Daniele et al. showed that AFP has been found to have a sensitivity of 41-65% and a specificity of 80-90% when detecting HCC given an AFP cut-off of 20 ng/mL [2].

Farinati et al. showed that up to 50% of patients with HCC have an AFP level below 20 ng/mL [3]. Johnson showed that elevated levels of AFP can also be found in patients with non-malignant chronic liver disease, including 15-58% with chronic hepatitis and 11-47% with liver cirrhosis [10]. Thus, AFP cannot be used as a sole tool to screen for and diagnose HCC. New reliable serum biomarkers need to be identified soon to complement AFP in order to improve clinical outcomes for patients.

The mean serum value of both AST and ALTin Egyptian HCC patients was significantly higher than those of the CHC patients followed by those of control group at (p<0.001) as shown in (Table 1). These results were in agreement with the studies of [16].

In the current study, there was a significant positive correlation between AFP and each of AST (r = 0.608) (r = 0.393) and ALT (r = 0.590) (r = 0.378), at P<0.01 in both HCC group and CHC group respectively.

It is now well known that both cancer initiation and neoplastic events are influenced by genetic background.

Control of cell proliferation is achieved by a precarious balance between regulation of apoptosis and cell cycle genes. BCL2 family members are key regulators of apoptosis, which is an important mechanism that plays a critical role in limiting viral replication in infected cells. BCL-2 is known to have anti-proliferative effects, by delaying progression to S phase from quiescence [5]. In addition, the anti-proliferative effect of BCL-2 has been shown to inhibit tumour progression in animal tumours [4].

BCL2 gene which is a member of the BCL2 family has anti-apoptotic activity and BCL2 gene has three genotypes; the Ala43Ala genotype, the Thr43Thr genotype, and the Ala43Thr genotype. The Thr43Thr and Ala43Thr genotypes of BCL2 gene were summed in 43Thr genotype.

Complete digestion of the wild type homozygote, gives; the Ala 43 genotype (157 bp+21 bp), mutant type homozygote, gives; the Thr43 genotype undigested (178 bp) and heterozygote, gives; the Ala 43 Thr genotype (178 bp+157 bp+21 bp) (Figure 6).

The frequencies of BCL-2 gene SNP at+127G/A in HCC patients, CHC patients and in healthy control individuals are summarized in (Table 3, Table 4 and Table 5).

The present study shows that healthy control individuals have a high frequency and non-significant difference of BCL-2 43 Ala genotype and allele compared with CHC patients(n=25, 83.3% vs n=21, 70%; n=55, 91.7% vs n=42, 70%, respectively).BCL2 Ala43Ala genotype of BCL2 gene may induce apoptosis which leads to cells homeostasis and induction in immune system. This mechanism sequence may leads to chronic hepatitis C infection elimination and inhibition of tumor progression.

The present study showed that, HCC patients who bear BCL-2 43 Thr genotype and allele also have a high frequency (about double) and significant difference compared with healthy controls (n=14, 46.6% versus n=5, 16.7%; n=18, 30% versus n=5, 8.3%, respectively) (P <0.05). This suggests that polymorphism in BCL-2 gene SNP at +127G/A can lead to predictors of loss of the suppressive effects of BCL-2 which may be advantageous for potentially malignant in CHC infected patients. So, BCL-2 43 Thr genotype and allele may be useful as a genetic biomarker for early prediction of susceptibility to the development of HCC in CHC patients.

The present results came in agreement with Huang et al., showed that the BCL-2 family plays a critical role in apoptosis. BCL-2 gene is a member of BCL-2 family and display anti-apoptotic activity [8].

In our study, table (6) showed that the frequency of 43 Thr allele and Ala 43 Thr, Thr 43 Thr, 43 Thr (Ala 43 Thr + Thr 43 Thr) genotype which show no significant difference between CHC patients and control groups (18.3% vs 8.3%, 23.3% vs 16.7%, 6.7% vs 0%, 30% vs 16.7%), Table (7) showed that the frequency of Thr 43 Thr was significally higher in HCC patient than that in control group (13.3% vs 0% at $p < 0.05$). Also, the frequency of 43 Thr (Ala 43 Thr + Thr 43 Thr) genotype was significally higher in HCC patient than that in control group (46.6% vs 16.7% at p <0.05). Also, the frequency of 43 Thr allele was significally higher in HCC patient than that in control group (30% vs 8.3% at p <0.05). So, 43 Thr (Ala 43 Thr + Thr 43 Thr) genotype was associated withthe risk of HCC prediction (46.6%, 4.375, 1.32 - 14.504, $p < 0.05$). Also, the 43 Thr allele was associated with the risk of HCC prediction (30%, 4.714, 1.618 - 13.732, $p < 0.05$). While the frequency of Ala 43 Thr genotype showed no significant difference between HCC patients and control group (33.3% vs 16.7%), and Table (8) showed that the frequency of 43 Thr allele and Ala 43 Thr, Thr 43 Thr, 43 Thr (Ala 43 Thr + Thr 43 Thr) genotype showed no significant difference between CHC patients and HCC patients (18.3% vs 30%, 23.3% vs 33.3%, 6.7% vs 13.3%, 30% vs 46.6%) that which may give information about risk factor for HCC development.

A search for mutations/polymorphisms in the human BCL-2 gene with association to autoimmunity, previously led to the identification of a single nucleotide polymorphism (SNP), G/A in the coding region of BCL-2 (exon 2) resulting in an Ala43Thr substitution. Results suggest that inhibition of the programmed cell death (apoptosis) function of 43Thr BCL-2 protein is suppressed compared with that of normal 43Ala BCL-2 protein. Komaki et al., 1998 showed that the polymorphism was reported to be frequent in 290 healthy control Japanese populations compared to a group of 221 Japanese T1DM patients (14.5% vs 6.8%) as well as to patients with other autoimmune diseases such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) (14.5% vs 8.0%).These findings suggest that this BCL-2 polymorphism, which is the first reported with association to T1DM, confers some resistance against T1DM and to autoimmunity in general [11].

On the contrary, a study searching for SNPs in the BCL-2 gene, using a panel of Mexican Mestizos and Swedish control subjects, did not find the Ala43Thr variation in these populations either [9]. It is likely that the Ala43Thr polymorphism arose spontaneously in Asians/Japanese after the human ancestor population separated into Caucasians and Asians in the early history of Man.

Overall, these observations and results encouraged us to establish the first study which examine the potential role of the BCL-2 genesingle nucleotide polymorphism at +127GNA (Ala43Thr) amongEgyptian CHC genotype (4) infected patients and HCC patients.

5. Conclusion

The present study investigated and demonstrated that Bcl2 gene polymorphism is associated with the susceptibility to HCC risk in Egyptian populations. Further large-scale studies are required to elucidate whether Bcl2 geneinteract with environmental factors in the development of HCC.

Acknowledgments

We want to express our appreciation to all the team workers in viral hepatitis research lab.

References

[1] Akkız, H., Bayram, S., Bekar, A., et al. A functional polymorphism in pre-microRNA-196a-2 contributes to the

susceptibility of hepatocellular carcinoma in a Turkish population: a case-control study. *J Viral Hepat*. 2011, 18:399-407.

[2] Daniele, B., Bencivenga, A., Megna, AS., et al. Alpha-fetoprotein and ultrasonography screening for hepatocellular carcinoma. *Gastroenterology* 2004, 127: S108-12.

[3] Farinati, F., Marino, D., De, Giorgio, M., et al. Diagnostic and prognostic role of alpha-fetoprotein in hepatocellular carcinoma: both or neither? *Am J Gastroentero* 2006, 101: 524-32.

[4] Furth, PA., Bar-Peled, U., Li, M., et al. Loss of anti-mitotic effects of Bcl-2 with retention of anti-apoptotic activity during tumor progression in a mouse model. *Oncogene* 1999, 18: 6589-96.

[5] Greider, C., Chattopadhyay, A., Parkhurst, C., et al. BCL-x(L) and BCL2 delay Myc-induced cell cycle entry through elevation of p27 and inhibition of G1 cyclin-dependent kinases. *Oncogene* 2002, 21: 7765-75.

[6] Hanafy, SM., Shehata, OH., Farahat, NM. Expression of apoptotic markers BCL-2 and bax in chronic hepatitis C virus patients. *Clinical Biochemistry* 2010, 43: 1112-1117.

[7] Holmes, JA., Desmond, PV. and Thompson, AJ. Does IL28B Genotyping Still have a Role in the Era of Direct-acting Antiviral Therapy for Chronic Hepatitis C Infection? *J Viral Hepat*. 2012, 19 (10): 677-684.

[8] Huang, D., Adams, J., Cory, S. The conserved N-terminal BH4 domain of BCL-2 homologues is essential for inhibition of apoptosis and interaction with CED-4. *EMBO J* 1998, 17:1029–39.

[9] Johansson, C., Castillejo-Lopez, C., Johanneson, B. Association analysis with microsatellite and SNP markers does not support the involvement of BCL-2 in systemic lupus erythematosus in Mexican and Swedish patients and their families. *Genes Immun* 2000, 1:380-5.

[10] Johnson, PJ. The role of serum alpha-fetoprotein estimation in the diagnosis and management of hepatocellular carcinoma. *Clin Liver Dis*. 2001, 5:145-59.

[11] Komaki, S., Kohno, M., Matsuura, N., et al. The polymorphic 43Thr BCL-2 protein confers relative resistance to autoimmunity: an analytical evaluation. *Hum Genet.* 1998, 103(4):435-440.

[12] Kvansakul, M. and Hinds, M G. Structural biology of the Bcl-2 family and its mimicry by viral proteins. *Cell Death Dis.* 2013, 4 (11): e909.

[13] Marcellin, P. Hepatitis C: the clinical spectrum of the disease. *J Hepatol*. 1999, 31:9–16.

[14] Mohamoud, YA., Mumtaz, GR., Riome, S., Miller, D. and Abu-Raddad, LJ. The epidemiology of hepatitis C virus in Egypt: a systematic review and data synthesis.*Bio Med Central Infectious Diseases* 2013, 13:288.

[15] Niederau, C., Lange, S., Heintges, T., et al. Prognosis of chronic hepatitis C: results of a large prospective cohort study. *Hepatology* 1998, 28: 1687–95.

[16] Seeff, LB. Natural history of hepatitis C. *Hepatology* 2002, 36(Suppl 1):S35–46.

[17] Song, P., Gao, J., Inagaki, Y., et al. Biomarkers: Evaluation of Screening for and Early Diagnosis of Hepatocellular Carcinoma in Japan and China. *Liver Cancer* 2013, 2:31-39.

[18] Taketa, K. Alfafetoprotein: reevaluation in hepatology. Hepatology 1990, 12: 1420-32.

[19] Tarr, AW., Urbanowicz, R A. and Ball, JK. The Role of Humoral Innate Immunity in Hepatitis C Virus Infection. *Viruses* 2012, 4(1): 1-27.

[20] Youssef, A., Yano, Y., Utsumi, T., abd El-alah, EM., abd El-HameedAel, E., SerwahAel, H., et al. Molecular epidemiological study of hepatitis viruses in Ismailia, Egypt. *Intervirology* 2009, 52 (3) 123-31.