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Association between Bone Morphogenic Protein-7 (BMP-7) Polymorphism (rs162316) and Chronic Liver Diseases among Egyptian Patients

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ABSTRACT

The bone morphogenic protein-7 (BMP-7) gene is a member of the transforming growth factor-β (TGF-β) superfamily. BMP-7 is believed to act as a regulator of endogenous control of hepatocytes proliferation and liver homeostasis in adult hepatocytes. The present study aimed to assess the association of single nucleotide polymorphism (SNP), rs162316 (G>A), in BMP-7 gene with the progression of HCV or HBV- chronic liver cirrhosis to hepatocellular carcinoma (HCC). The tetra-primer amplification-refractory mutation system (ARMS-PCR) method was used for rs162316 genotyping. In this study, 150 subjects were divided into 50 healthy controls, 50 HCV or HBV-cirrhotic patients with HCC and 50 HCV or HBV-cirrhotic patients and HCC-free. Results of rs162316 genotyping revealed an association of SNP with 1.14 (0.64-2.03) and 1.43(0.79-2.58) alleles A frequencies (OR, CI 95%) in HCC patients compared to cirrhotic and healthy control groups respectively. For the specific studied SNP of BMP7 gene rs162316, the A allele (AG genotype) and was significantly associated with HCC progression. However, AG genotype of rs162316 may be considered as a predictor for HCC in Egyptian cirrhosis patients.

Keywords: Bone morphogenic protein-7, Single nucleotide polymorphism, Hepatocellular carcinoma, Liver cirrhosis.

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1. INTRODUCTION

There are many liver diseases that reduce liver functions. Some liver diseases are associated with increased risk for cancerous tumors like hepatoma, including cirrhosis, fatty liver disease (FLD) **(Feldman et al**., **2006)**. Cirrhosis is a form of chronic liver disease (CLD) resulting from sustained liver damage from a number of causes. Liver cirrhosis is mainly caused by the infection with hepatitis B or C viruses, autoimmune hepatitis, alcohol misuse and cardiac hepatopathy **(Muir, 2015)**. HBV infection is a common increasing health problem in developing countries, such as Egypt, which is considered as an intermediate endemic country. The prevalence of HBsAg ranges from 2-8 % of the population, nearly 2-3 million Egyptians are chronic carriers of HBV **(El-Zayadi, 2007).** Hepatitis C virus (HCV) constitutes a significant health burden worldwide, too. In the long-term, this can lead to advanced liver fibrosis, cirrhosis and hepatocellular carcinoma **(Kandeel et al., 2017).** However, hepatocellular carcinoma (HCC) is one of the most common malignant tumors worldwide. It represents the fifth most malignant tumor worldwide and the third most common cause of cancer mortality. It is thought to be attributed to cumulative genetic alterations **(Padma et al., 2009).**

Bone morphogenic protein-7 (BMP-7), also known as osteogenic protein-1 or (OP-1), is a member of the transforming growth factors β (TGF-β). This superfamily in humans is encoded by the BMP-7 gene. Itis produced by osteoblasts and other bone cells. It affects osteoblast proliferation and differentiation and control of multiple key steps of cell growth, migration, and apoptosis in various types of cancers **(Tang et al., 2013).** BMP-7 is not only expressed in various normal tissues including bone, brain, spleen, skeletal muscle, kidney and liver, but also in many types of cancers such as lung cancer, gastric cancer, melanoma, breast cancer, and colorectal cancer **(Lu et al., 2014).**

Recently, it has become obvious that BMP-7 is a very pleiotropic growth factor. It plays an essential role in the development of bone and kidney. Also, it controls the development and maintenance of many physiological processes in the human body. Consequently, its aberrant expression of BMP is associated with many diseases such as incomplete fracture healing, osteoarthritis, the development of bone metastases and renal fibrosis **(Boon et al., 2011).**

Nowadays, single nucleotide polymorphisms (SNPs) are considered to be the most critical genetic predisposition factors in humans to be susceptible to diseases **(El-Nabi et al., 2019)**. Furthermore, they are assumed to have an important role in the severity of the disease and the response to the treatment as well **(El-Garawani et al., 2020)**. A previous study supported the role of genetic variation in BMPs in the etiology of breast cancer. However, the BMP-7 gene polymorphism (rs7273197) was associated with breast cancer in the intermediate ancestry group **(Slattery et al., 2013).** Single nucleotide polymorphisms (SNP's) in the BMP-7 gene may predict impaired fracture healing. Knowledge of these SNP's may minimize fracture morbidity by altering the initial fracture treatment and yield further insight into the molecular genetics of fracture healing **(Sathyendra et al., 2012).**

The current study is concerned with specific rs162316 SNP which is located in 20 q13.31(BMP-7, intron- 1). Consequently, this study was conducted to investigate the association of a specific SNP of BMP-7 gene (rs162316) with the development of HCC from liver cirrhotic Egyptian patients.

2. SUBJECTS AND METHODS

2.1. Subjects

The study population consisted of 150 individuals who were divided into three groups. The first group (HCC group) was comprised of 50 patients with liver cirrhosis and HCC caused by HCV or HBV infections. The second group (cirrhosis group) consisted of 50 patients with liver cirrhosis without HCC. The third group (control group) was comprised of 50 healthy controls.

The study plan was reviewed and approved by the Ethical Committee at National Liver Institute, Menoufia University (No: 00139/2018). The study was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines. The patients enrolled were from the Outpatient Unit of Hepatology and Gastroenterology Unit of National Liver Institute Hospital, Menoufia University, Egypt. Informed consent was obtained from all participants included in the study.

2.2. Methods

2.2.1. Samples collection

Peripheral venous blood samples were collected using two types of tubes, the sterile K_2-EDTA-containing tubes (KEMICO vacutainer, Egypt), for clinical lab examination of complete blood count (CBC) and for the isolation of peripheral blood leucocytes. The other type of tube is the sterile serum plain tubes (KEMICO vacutainer, Egypt) for the estimation of liver function tests [Alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin, total and direct bilirubin and alpha feto protein (AFP, as a tumor marker)]. All samples were labeled by numbers which were matching with the numbers of other investigations.

2.2.2. Isolation of peripheral blood leucocytes

About two milliliters of peripheral venous blood samples were incubated with five volumes of erythrocyte lysing buffer (0.015M NH_4C1, 1mM $NaHCO_3$, 0.l mM EDTA). Then, they were centrifuged for 5 minutes at 1000 rpm using a cooling centrifuge (Sigma 3K30, Germany). These steps were repeated until a white pellet appeared **(El-Garawani 2015).**

2.2.3. Total genomic DNA isolation

Genomic DNA was isolated from the peripheral blood leukocytes according to the "salting out extraction method. Proteins were precipitated from the tissue lysate solution using a solution of NaCl (4M) **(Aljanabi and Martinez, 1997).**

2.2.4. Genotyping

Tetra primers ARMS-PCR method was carried out to investigate the SNP (rs162316) polymorphism in the BMP-7 gene. All amplifications were performed in a thermo-cycler Master cycler gradient (Eppendorf, Germany). DNA samples were processed for initial denaturation at 94^{o}C for 10 min, followed by 35 cycles of denaturation at 94^{o}C, annealing at 62^{o}Cand extension at 72^{o}C for 1 min. Primers sequences are shown in Table (1). The fragments size was chosen within the range of 300–705 bp. The A allele product appeared at 385 bp and G allele at 300 bp. The amplification reactions were carried out using the four primers in one reaction tube at the same time.

The PCR products were separated on 2.5 % agarose gels (Sigma-Aldrich, Germany) and stained with ethidium bromide for visualization on UV trans-illuminator **(El-Garawani and Hassab El Nabi, 2016).**

Table (1): The oligonucleotide primers used in rs162316 genotyping.

Primer	Sequences
Outer forward	5′- CATAGCTCATCAGGATGAGCAAAGT -3′
Outer reverse	5′- CATCTGGAGAGAACACAGCTACTGG -3′
Inner forward	5′- GAAAGAAGCTTCCAAGCCCG -3′
Inner reverse	5′- CAATTCCAACCTCAGTCCGCT -3′

2.2.5. Statistical analysis

Results were statistically analyzed by SPSS version 22 (SPSS Inc., Chicago, IL, USA). For descriptive data, percentage (%), mean and standard deviation SD were determined. Analytical data indicate the presence of any significant difference between several groups for a normally distributed quantitative variable. They have been analyzed using one way ANOVA (F test) and Kruskal -Wallis test which is the non-parametric version of ANOVA. For showing any significant difference between the individual groups, the Post hoc test was used. To compare two groups, or more, regarding one qualitative variable in a 2x2 contingency table or r c complex table was analyzed using Chi-Squared ($\chi 2$). Pearson's Correlation analysis was used for showing strength and direction of the association between two quantitative variables. The Receiver operating characteristic (ROC curve) is a comparison of two operating characteristics; true positive rate (TPR) &false positive rate (FPR). It was used to check the level of sensitivity and specificity of AFP to identify its role as a marker for diagnosis and detection of liver cirrhosis and HCC. The odds ratio and the 95% confidence interval (CI) were calculated for the proportion of genes and alleles among the studied groups. *P* values < 0.05 were considered as statistically significant and *p* values < 0.005 were considered as statistically highly significant.

3. RESULTS

The demographic data of the HCC, cirrhosis and control groups showed that there was no significant (*P*> 0.05) difference between the groups studied regarding age or sex (Table. 2). Serum levels of AFP were significantly higher among all patient groups than the healthy control group with (*P*<0.001). However, the highest levels were reported in HCC patients (928.57 ±840.47) followed by cirrhosis group (8.32 ±7.38) compared with their levels in control (4.02±1.41) (Table. 3). The Receiver operating characteristic curves (ROC curves) have been compared to all patients with controls AUC for AFP (0.85) with higher specificity 68%, sensitivity 81%, and accuracy 77 % when reading diagnosis of HCC with AFP at a cutoff point of 113.50. They had the upper hand as well positive and negative tests (Table. 4).

Table (2): Demographic data of the groups studied (HCC, Cirrhosis and control):

	Patients							
	HCC (n=50)		**Cirrhosis (n=50)**		**Controls (n=50)**		**Test of sig**	**P value**
Age(Y) Mean ±SD	60.28±6.76		56.06±8.18		59.12±13.18		F=2.49	0.086
Sex: no,%							χ^2	
Male	38	76.0	33	66.0	30	60.0	2.97	0.226
Female	12	24.0	17	34.0	20	40.0		

F: ANOVA, χ^2: Chi-Square, * Significant

Table (3): Alpha feto protein (AFP) levels among the groups studied

	Patients				
	HCC (n=50)	**Cirrhosis (n=50)**	**Controls (n=50)**	**Kruskal-Wallis test *P* value**	**Post hoc**
	Mean ±SD	Mean ±SD	Mean ±SD		
AFP (ng/ml)	928.57 ±840.47	8.32 ±7.38	4.02 ±1.41	105.23 <0.001**	P1, P2, P3 <0.001**

* Significant, ** highly significant, P1: HCC versus cirrhosis, P_2: HCC versus controls, P_3: Cirrhosis versus controls

Table (4): Specificity and sensitivity of AFP for patients (HCC and Cirrhosis)

Variable	AUC	Cutoff point	Sensitivity %	Specificity %	Accuracy %	PPV %	NPV %
All patients							
• AFP	0.85	>4.25	81.0	68.0	77.0	84.0	64.0
• **Combined**	-	-	99.0	34.0	77.0	75.0	94.0
HCC							
• AFP	1.0	113.50	100.0	100.0	100.0	100.0	100.0
Liver cirrhosis							
• AFP	0.71	>3.75	74.0	52.0	63.0	61.0	67.0

AUC: Area under curve, PPV: Positive predictive value, NPV: Negative predictive value.

The specific laboratory investigations among the groups studied showed that liver function tests including ALT, AST, Direct Bilirubin and Total Bilirubin were significantly higher among patients groups than control (P<0.001). However, within patient groups, AST, direct bilirubin and total bilirubin were significantly higher among the HCC group than the cirrhosis group (P<0.05). In contrast, albumin was significantly lower in all patient groups than control (P<0.001). Also, haematology parameters including Hb, WBCs, and platelets were significantly lower in all patient groups than control (P<0.001). Within patient groups, platelets counts were significantly lower in HCC-patients than cirrhotic patients (P<0.05) as shown in Table (5).

Table (5): Specific laboratory investigations among the groups studied

	Patients				
	[1]HCC (n=50)	**[2]Cirrhosis (n=50)**	**[3] Controls (n=50)**	**Kruskal-Wallis test *P* value**	**Post hoc**
	Mean ±SD	Mean ±SD	Mean ±SD		
ALT (U/L)	57.70 ±28.13	49.72 ±19.26	30.66 ±10.47	40.63 <0.001**	P2,P3 <0.001**
AST (U/L)	58.64 ±24.50	48.86 ±19.67	28.08 ±8.90	57.61 <0.001**	P1<0.05* P2,P3 <0.001**
Direct Bilirubin (mg/dl)	0.72 ±0.51	0.54 ±0.50	0.19 ±0.06	69.91 <0.001**	P1<0.05* P2,P3 <0.001**
Total Bilirubin (mg/dl)	1.51 ±0.78	1.25 ±0.78	0.58 ±0.12	71.24 <0.001**	P1<0.05* P2,P3 <0.001**
Albumin (g/dl)	3.33 ±0.58	3.46 ±0.64	4.34 ±0.45	F=46.31 <0.001**	P2,P3 <0.001**
Haemoglobin (Hb) (g/dl)	11.90 ±1.11	11.76 ±1.09	12.81 ±1.29	F=11.70 <0.001**	P2,P3 <0.001**
White Blood Cells (WBCs) (10^3/mm^3)	5.14 ±1.90	4.72 ±1.69	7.25 ±1.98	38.58 <0.001**	P1=0.918 P2,P3 <0.001**
Platelets (10^3/mm^3)	122.28 ±39.57	143.54 ±37.61	231.96 ±59.89	75.24 <0.001**	P1<0.05* P2,P3 <0.001**

* Significant, ** highly significant, P1: HCC versus Cirrhosis, P2: HCC versus Controls, P_3: Cirrhosis versus controls.

In order to perform rs162316 genotyping, the PCR products electrophoresis was done on agarose gel (Figure.1). The allele frequencies of rs162316 were illustrated in Table (6). Results showed that AA and GG have reported a prevalence of 10% and 34% among HCC groups respectively, versus 14% and 44% respectively among the cirrhosis group. Records among controls were 18% and 58% respectively. The AG genotype reported higher prevalence among HCC (56%) than cirrhosis (42%) and controls (24%) with *P*=0.004.

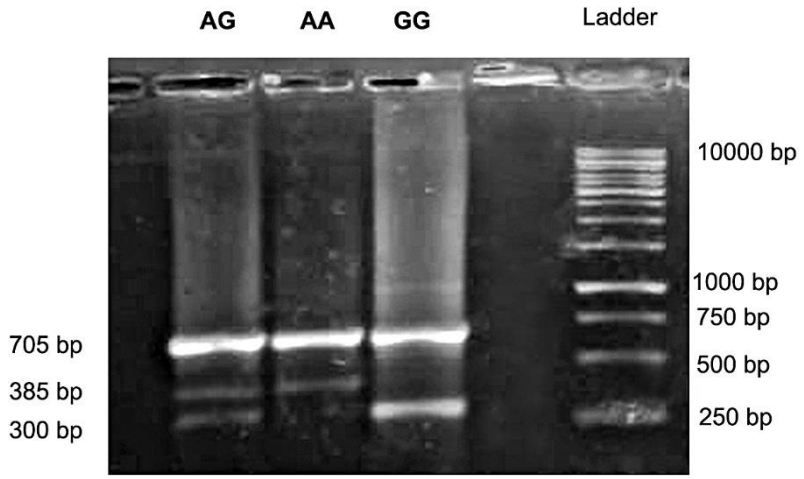

Figure (1): Representative digital photograph of ARMS-PCR amplified products resolved on 1.5% agarose gel electrophoresis showing the BMP-7 (rs162316) genotyping against GeneRuler 1 kb DNA ladders (Thermo Scientific, O'Gene Ruler™, US).

Table (6): The genotype distribution and allele frequencies of rs162316 R [A/G] among the groups studied:

	Patients							
	HCC (n=50)		**Cirrhosis (n=50)**		**Controls (n=50)**			***P* value**
	no	**%**	**no**	**%**	**no**	**%**	χ^2	
Genotype							**10.67**	**P=0.030**
GG	17	34.0	22	44.0	29	58.0	1.97	P_1=0.372
AG	28	56.0	21	42.0	12	24.0	10.67	P_2=0.004*
AA	5	10.0	7	14.0	9	18.0	3.67	P_3=0.159
Alleles frequency							0.19	P_1=0.659
G	62	62.0	65	65.0	70	70.0	1.43	P_2=0.232
A	38	38.0	35	35.0	30	30.0	0.57	P_3=0.450

*Significant, ** Highly Significant, Chi-Squared (χ^2), P: between all groups, P_1: HCC versus Cirrhosis, P_2: HCC versus Controls, P_3: cirrhosis versus controls

The genotypic odds ratio and 95% CI of rs162316 R [A/G] genotype distribution and allele frequencies among all the groups studied showed that "AG" genotype of rs162316 was significantly higher among HCC patients (56%) than healthy controls (24%) making those who carry this genotype to be nearly 4 times more risky to have HCC than controls (OR=3.98, CI: 1.61-9.82, *P*=0.002. (Table. 7).

Table (7). The genotypic Odds ratio and 95% CI of rs162316 R [A/G] genotype distribution and allele frequencies among the groups studied.

	Groups						
	HCC (n=50)		**Cirrhosis (n=50)**		χ^2	***P* value**	**OR (CI 95%)**
	no	**%**	**no**	**%**			
Genotype							
GG (wild type)	17	34.0	22	44.0			**1.0**
AG	28	56.0	21	42.0	1.60	0.206	1.73(0.74-4.03)
AA	5	10.0	7	14.0	0.01	0.906	0.92(0.25-3.43)
Alleles frequency							
G	62	62.0	65	65.0			**1.0**
A	38	38.0	35	35.0	0.19	0.659	1.14(0.64-2.03)
	HCC (n=50)		**Controls (n=50)**				
Genotype							
GG	17	34.0	29	58.0			1.0
AG	28	56.0	12	24.0	9.36	0.002*	3.98(1.61-9.82)
AA	5	10.0	9	18.0	0.01	0.932	0.95(0.27-3.30)

Alleles frequency							
G	62	63.0	70	70.0			1.0
A	38	38.0	30	30.0	1.43	0.232	1.43(0.79-2.58)
	Cirrhosis (n=50)		**Controls (n=50)**				
Genotype							
GG	22	44.0	29	58.0			**1.0**
AG	21	42.0	12	24.0	3.37	0.066	2.31(0.94-5.67)
AA	7	14.0	9	18.0	0.0	0.965	1.03(0.33-3.18)
Alleles frequency							
G	65	65.0	70	70.0			**1.0**
A	35	35.0	30	30.0	0.57	0.450	1.26(0.69-2.27)

Chi-Squared (χ^2), (OR) Odds ratio*Significant, **highly significant.

4. DISCUSSION

BMPs are extracellular signaling molecules and are the largest group of the TGFβ superfamily. Because of their role in cell regulation, proliferation, apoptosis, and migration, they have been implicated as potentially important in cancer etiology **(Slattery et al., 2013).**

The present study aimed to investigate the association of a specific single nucleotide polymorphism of BMP-7 gene (rs162316) with the development of liver cirrhosis disease to HCC caused by HCV or HBV infection. Results of this study found that HCC and cirrhotic patients had high levels of AFP compared to controls supporting the previous studies of **Steward and Rosenfeld (2008)** and **Tai et al. (2009)**. The elevated AFP levels are observed also in cirrhosis without HCC which was in agreement with a study done by **Di Bisceglie (2005**) who explained the AFP increasing due to inflammation, necrosis and hepatocellular injury

which possibly resulted from increased hepatocyte turnover.

In the current study, the validity and cutoff points of BMP-7 in the diagnosis of cirrhosis provided the evidence that a high-expression level of BMP-7 serves as a biomarker for prognosis for liver cirrhosis. On the other hand, the diagnosis of HCC, AFP at cutoff point of (113.50) had a sensitivity of (100%) and specificity of (100%) with the upper hand as good positive and negative test. These results are in agreement with a study done by **Yvamoto et al. (2015)** who found that AFP had a sensitivity of (28%) and specificity of (99%),using a cutoff value of 200 ng/ml for differentiation between HCC and liver cirrhosis.

The genetic variation in BMP-7 with different SNPs was discussed in many previous studies which revealed an association between BMP-7 gene and distinct diseases. A previous study showed rs6127921; a significant association with treatment response to selective serotonin reuptake inhibitors (SSRIs) in major depressive disorder **(Esaki et al., 2013)**. This is similar to the study done Martha et al who supported the role of genetic variation in BMPs in the etiology of breast cancer. Associations of BMPs related SNPs were, in some instances, influenced by menopausal status and resulted in associations that were specific to estrogen and progesterone receptor status of tumors. BMP-7 gene genotyping of rs7273197 supported the association with breast cancer **(Slattery et al., 2013).**

In the current study, genotyping of rs162316 revealed that heterozygous (AG) genotype revealed a significant genetic variation in HCC patients. It is nearly 4 times more risky to acquire HCC than healthy controls. These result may be due to the over-expression of BMP-7 in HCC cells than in normal hepatic cells and related to tumor size which considered the BMP-7 as an oncogene in HCC **(Li et al., 2013).** Contrarily, AG genotype of rs162316 showed no significance between control and any group of patients to determine the association of BMP-7 polymorphisms and BMD and osteoporotic fracture in postmenopausal Chinese women **(Gao et al., 2016)**.

5. CONCLUSION

The study showed that AG genotype (rs162316) revealed a genetic association with HCC cases where it was nearly 4 times more risky to acquire HCC than healthy controls.

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