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Original Research Article

Relationship between Vascular Endothelial Growth Factor Polymorphism (Rs699947) and the Development of Hepatocellular Carcinoma

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Abstract

Background: Angiogenesis is defined as the expansion and remodeling process affecting the vascular network and it occurs in many pathological conditions including cancer. Hepatocellular carcinoma (HCC) is one of the hyper-vascular cancers. Understanding the process of angiogenesis and its regulatory mechanisms are crucial in HCC treatment. Almost all approved systemic therapies used in HCC target the angiogenesis process especially the vascular endothelial growth factor (VEGF) pathway. Studying genetic variations and other factors that affect angiogenesis could allow tailoring systemic therapy with the most benefits to patients. ***Aim***: Investigating the association between the VEGF variant (rs699947) and the progression of HCC was the main target of this study. ***Methods***: A total of 122 subjects were enrolled in this study (64 patients with HCC, 24 patients with cirrhosis and 34 subjects served as a control group). Genotyping of the VEGF gene (rs699947) was performed by tetra primer amplification refractory mutation system-polymerase chain reaction (ARMS-PCR). ***Results***: The AA genotype and the A-allele were found to be lower in patients with HCC compared with other groups. In HCC patients, the AA genotype was associated with higher serum albumin and lower total bilirubin level. ***Conclusion***: The variant allele (rs699947) could be considered as a predictive factor for HCC development.

Keywords: Vascular endothelial growth factor, Angiogenesis, Hepatocellular carcinoma, rs699947, polymorphism.

INTRODUCTION

Hepatitis C virus (HCV) infection is associated with high morbidity and mortality, so it has great attention all over the world. Its burdens lie heavily particularly on countries with low income [1]. Although there is a global decline in HCV prevalence, its complications are rising due to liver fibrosis progression and the aging of the infected population [2]. Hepatocellular carcinoma (HCC) represents an international public health concern as one of the most common cancers worldwide. It is the fourth most common cause of cancer-related death worldwide and is the sixth regarding incidence rate [3]. HCC is a characteristically hyper-vascular tumor. This feature helps in both the diagnosis and treatment of this type of cancer. The presence of abundant and tortuous vessels distinguishes HCC from other benign liver conditions in angiography and imaging. Chemoembolization is the standard of care for intermediate-stage HCC and drugs targeting angiogenesis are also effective [4]. Angiogenesis is the process of new blood vessel creation from the already existing ones. Angiogenesis is a very crucial process in both physiological and pathological conditions including tumor growth, and it is firmly regulated by several factors [5]. Many pathological conditions, including tumors, can lead to uncontrolled angiogenesis. Tumor-induced angiogenesis is enabled by over-expression of proangiogenic factors, along with under-expression of anti-angiogenic factors, causing increased tumor vascular density with abnormal vascular structure [6]. The VEGF is among the most common pro-angiogenic factors. There are four VEGF isoforms (A, B, C, and D) in addition to the placental growth factor (PlGF) encoded by related different genes. VEGFs are known to be regulated by many viruses including HCV and hepatitis B virus (HBV) infection, which constitutes a major risk for HCC development. The progress of HCV and HBV into HCC is mediated by chronic

inflammation and induction of angiogenesis mainly through the VEGF up-regulation. The HCV core protein and the hepatitis B viral protein x (HBx) are the main mediators of HCV and HBV-dependent VEGF up-regulation respectively [7].

The VEGF has a highly polymorphic gene. The genetic variations in VEGF are suggested to be associated with the development and progression of many diseases and cancers [8, 9]. They are also important in the field of pharmacogenetics as they influence the response to many therapeutic agents. These facts encouraged the study of the effect of these variations on HCC. One of these variations is the rs699947 which lies in the promotor region and is assumed to influence VEGF expression level [10]. In this study, we aimed to explore the association of VEGF gene polymorphism (rs699947) among Egyptian liver cirrhosis and HCC patients. The study findings can possibly predict the HCC occurrence and progression from liver cirrhosis.

SUBJECTS AND METHODS

Subjects

This study was conducted on 122 subjects. They were 64 patients with HCC, 24 patients with cirrhosis with no radiological evidence of HCC, and 34 apparently healthy subjects who were served as a control group. The three groups were gender-matched. The study plan was reviewed and approved by the Ethical Committee at Menoufia University (No: 00139/2018). The study was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines. Patients were enrolled from the National Liver Institute, Menoufia University, Egypt. Informed consents were obtained from all participants included in the study. The inclusion criteria of patients with HCC were adults above 18 years, both male and female were enrolled, confirmed diagnosis by imaging methods as Triphasic computed tomography (CT) or Magnetic resonance imaging (MRI). HCC was diagnosed by a characteristic pattern of vascular enhancement detected on multislice triphasic spiral CT scan and/ or MRI according to the established diagnostic criteria [11]. The exclusion criteria for the involved patients were the unconfirmed diagnosis or those with tumors other than HCC. The diagnosis of liver cirrhosis was based on history taking, clinical examination and abdominal ultrasound examination. The Child–Pugh score was used for assessing the severity of liver cirrhosis [12].

Clinical and laboratory tests

Relevant clinical data were collected. Samples of peripheral venous blood were collected using EDTA anticoagulant-containing tubes and other aliquots for serum isolation. Basic laboratory tests were done including complete blood count using Sysmex xp-300AM automated hematology analyzer (Sysmex Corporation, Kobe, Japan), liver function tests including [alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin, direct bilirubin and albumin], α-fetoprotein (AFP) and hepatitis serology (hepatitis B surface antigen [HBsAg] and hepatitis C virus antibodies [HCV Ab]) using Cobas C501, e501 module autoanalyzer (Roche Diagnostics, Mannheim, Germany).

Molecular testing

Peripheral blood leucocytes were isolated from EDTA anti-coagulated blood samples, within four hours of collection using the erythrocyte lysing buffer (0.015 M NH_4C1, 1 mM $NaHCO_3$, 0.1 mM EDTA) method [13]. The isolation of genomic DNA from the leukocytes was performed by salting out extraction method [14]. Genotyping for rs699947 was performed by tetra-primer amplification refractory mutation system polymerase chain reaction (ARMS-PCR). The primers used were Forward inner primer (A-allele): 5'CAGCTGTAGGCCAGACCCTGGTAA3', Reverse inner primer (C-allele): 5'TCAGTCTGATTATCCACCCAGACCG3', Forward outer primer: 5'CAGCCCTTTTCCTCATAAGGGCC3' and Reverse outer primer: 5'TCCCTAAGTGCTCCCAAAGGCC3'. All amplifications were performed in a thermo-cycler Master cycler gradient (Eppendorf, Germany). DNA samples were processed for initial denaturation at 94°C for 10 min, followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1min and extension at 72°C for 45 sec, the final extension was done for 5 min at 72°C. A total of 5µl of the products were resolved on 2.5 % ethidium bromide-stained agarose gel (Sigma-Aldrich, Germany). The results were observed under UV light and digital photographs were captured. Product size for A and C alleles were 198 and 250bp respectively, however, the two outer primers produced an amplicon with a 399bp.

STATISTICAL ANALYSIS

Results were collected, tabulated and statistically analyzed with the statistical package SPSS version 20 (SSPS, Inc., Chicago, IL, USA). Differences in allele frequencies and genotype distribution between the studied groups were weighed by Pearson χ^2 test. Odds ratios and 95% Confidence Interval were calculated. For the comparison of clinical and laboratory variables with the distribution of different genotypes, the Chi-square (χ^2) test was used for qualitative data and analysis of variance (ANOVA (F)) or Kruskal–Wallis (k) for quantitative data. Statistical significance was considered when the P-value was < 0.05.

RESULTS

Demographic and clinical characteristics

The demographic, clinical and basic laboratory data of the studied subjects are demonstrated in Table 1. There was no significant difference among the three

studied groups regarding gender (p=0.75) or child score (p= 0.06).

Table-1: The demographic, clinical and basic laboratory data of the studied subjects

Groups Variables	Group 1 (Control) n=34		Group2 (Cirrhosis) n=24		Group3 (HCC) n=64		Test	P value
	No	%	No	%	No	%		
Age (years) (Mean±SD)	40.9±7.3		54.9±6.7		59.6±7.0		F = 78.90	-
Gender: Female Male	12 22	35.3 64.7	6 18	25 75	16 48	25 75	χ^2 27.21	0.750
Hb (g/dl) (Mean±SD)	13.0±1.1		11.8±0.89		11.9±0.94		F= 15.77	< 0.001
WBCs×10^3 (cell/mm^3) (Mean±SD)	7.3±1.8		3.9±0.9		5.2±1.8		F= 29.39	< 0.001
Platelets×10^3 (cell/mm^3): Mean±SD Median	236.78±56.45 220.00		125.12±30.64 130.00		132.9±70.7 120.00		K = 58.3	< 0.001
ALT(IU/dl) Mean±SD Median	30.9±10.64 29.0		57.2±17.2 62.0		59.5±28.4 51.0		K =19.214	< 0.001
AST(IU/dl) Mean±SD Median	27.6±10.1 28.0		57.3±20.2 59.0		60.8±26.6 50.0		K =27.726	< 0.001
Albumin (gm/dl): Mean±SD	4.4±0.4		3.5±0.6		3.4±0.6		F = 40.046	< 0.001
Total Bilirubin (mg/dl) Mean±SD Median	0.5±0.1 0.6		1.3±0.9 1.1		1.4±0.6 1.3		K=21.53	< 0.001
Direct Bilirubin (mg/dl) Mean±SD Median	0.1±0.05 0.1		0.62±0.58 0.4		0.65±0.38 0.6		K =17.56	< 0.001
AFP (ng/dl) Mean±SD Median	3.8±1.00 3.7		13.2±16.4 4.2		609.1±1245.2 24		K =6.54	0.002
HBsAg: Positive Negative	-------		3(12.5) 21(87.5)		6(9.4) 58(90.6)		χ^2= 0.186	0.667
HCV-Ab: Positive Negative	--------		22(91.7) 2(8.3)		59(92.2) 5(7.8)		χ^2= 0.006	0.936
Edema: Present Absent	-------		10(41.7) 14(58.3)		30(46.9) 34(53.1)		χ^2= 0.191	0.662
Ascites: Present Absent	--------		6(25) 18(75)		13(20.3) 51(79.7)		χ^2=0.227	0.634
Jaundice: Present Absent	--------		13(54.2) 11(45.8)		19(29.7) 45(70.3)		χ^2=4.52	0.034
Child score A B C	-----		15(62.5) 7(29.2) 2(8.3)		41(64.1) 23(35.9) 0(0)		χ^2=5.57	0.06

SD= Standard deviation, Hb= Hemoglobin, WBCs= White blood cells, ALT= Alanine aminotransferase, AST= Aspartate aminotransferase, AFP= α-fetoprotein, HBsAg= Hepatitis B surface antigen, HCV Ab= Hepatitis C virus antibody, F = One-Way ANOVA test, K= Kruskal–Wallis, χ^2= Chi-Square test.

Genotyping of rs699947

The allelic frequency of rs699947 was assessed using tetra primer ARMS-PCR, and the products were resolved on agarose gel (Figure 1). The current study revealed a statistically significant difference between HCC, cirrhotic and control groups regarding rs699947 genotypes (Dominant comparison p<0.001 and recessive comparison, p=0.009). HCC

group had lower frequency for the A allele compared to cirrhotic group (p <0.001, OR = 0.20, 95% CI =0.10-0.40) and control group (p=0.008, OR = 0.44, 95% CI =0.24-0.81). Moreover, there was a significant difference between the cirrhotic and the control groups regarding rs699947 genotypes and allele frequencies (Dominant comparison p=0.018, OR = 6.00, 95% CI =1.20-30.00 and allele comparison p=0.036, OR =2.25, 95% CI = 1.05-4.84) as shown in Table 2.

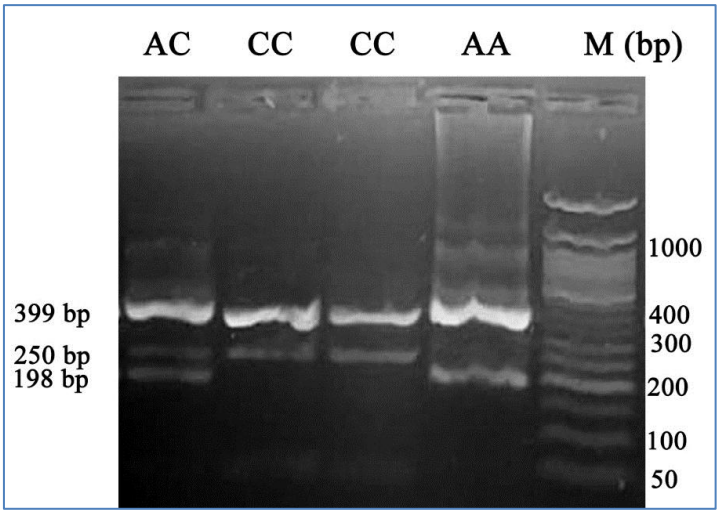

Fig-1: Representative digital photograph of tetra primer ARMS-PCR products resolved on 2.5% ethidium bromide-stained agarose gel electrophoresis showing the VEGF (rs699947) genotyping; M, O'Gene Ruler™ 100 bp DNA ladders (Thermo Fisher Scientific, Austin, TX, USA)

Table-2: Comparison between control, cirrhotic and HCC groups regarding genotype rs699947.

	Control (n=34) N (%)	Cirrhotic (n=24) N (%)	HCC (n=64) N (%)	χ^2	P value	Odds ratio (95% confidence interval)
Genotype rs699947					0.001[a]	
CC	12 (35.3)	2 (8.3)	36 (56.2)		0.000 [b]	
AC	12 (35.3)	12 (50.0)	20 (31.2)	19. 18[a]	0.062 [c]	
AA	10 (29.4)	10 (41.7)	8 (12.6)		0.061 [d]	
Dominant comparison					<0.001[a]	
AA+AC					<0.001[b]	0.07 (0.02-0.33) [b]
CC	22 (64.7)	22 (91.7)	28 (43.8)	17.2[a]	0.048[c]	0.42 (0.18-1.0) [c]
	12 (35.3)	2 (8.3)	36 (56.2)		0.018[d]	6.00 (1.20-30.00) [d]
Recessive comparison				9.51[a]	0.009 [a]	
CC+AC	24 (70.6)	14 (58.3)	56 (87.4)		0.006 [b]	0.20(0.07-0.6) [b]
AA	10 (29.4)	10 (41.7)	8 (12.6)		0.044 [c]	0.34(0.12-0.97) [c]
					0.335[d]	1.71(0.57-5.13) [d]
Allele frequency					<0.001[a]	
C	36 (52.9)	16 (33.3)	92 (71.9)	22.88[a]	<0.001[b]	0.20 (0.10-0.40) [b]
A	32 (47.1)	32 (66.7)	36 (28.1)		0.008 [c]	0.44 (0.24-0.81) [c]
					0.036 [d]	2.25 (1.05-4.84) [d]

χ^2= Chi-Square test, [a]between the three groups, [b]Between HCC and cirrhotic, [c] Between HCC and control, [d]Between cirrhotic and control.

In patients with HCC, the A/A genotype showed significantly higher serum albumin and lower total bilirubin compared with the C/C and A/C genotypes (P=0.015 and 0.048 respectively). No significant difference was found between the studied groups regarding other clinical and laboratory parameters (Table 3).

Table-3: Analysis of liver function and α-fetoprotein according to rs699947 genotype in HCC group

	CC N=36	AC N=20	AA N=8	Test	P value
ALT(U/L)				K= 1.042	0.359
Range(Min-Max)	19-113	22-121	29-92		
Mean ±SD	58±27	65±32	48±27		
AST(U/L)				K= 1.157	0.321
Range(Min-Max)	28-125	25-117	35-78		
Mean ±SD	63±25	62±30	47±18		
Albumin(g/dl)				F= 4.514	0.015
Range(Min-Max)	2.1-4.1	2.9-4.2	2.8-4.6		
Mean ±SD	3.2±0.5	3.5±0.5	3.8±0.7		
Total bilirubin(mg/dl)				K=3.193	0.048
Range(Min-Max)	0.8-3.2	0.6-1.9	0.7-1.4		
Mean ±SD	1.5±0.6	1.2±0.4	1.1±0.2		
AFP (ng/ml)				K= 1.246	0.295
Range(Min-Max)	3.5-4415	3.3-4415	7.5-376		
Mean ±SD	1292±802	1353±456	163±111		
Child Pugh				χ^2=1.258	0.53
A	21 (58.3)	14 (70)	6 (75)		
B	15 (41.7)	6 (30)	2 (25)		
Ascites				χ^2=0.127	0.94
present	7 (19.4)	4 (20)	2 (25)		
absent	29 (80.6)	16 (80)	6 (75)		
Jaundice				χ^2=2.21	0.33
present	10 (27.8)	8 (40)	1 (12.5)		
absent	26(72.2)	12 (60)	7 (87.5)		

F = One-Way ANOVA test, K= Kruskal-Wallis, χ^2= Chi-Square.

DISCUSSION

The incidence of HCC is rising all over the world due to the rise in the incidence of HBV and HCV infections [15]. The polymorphisms in VEGF had gained much interest for their potential role in predicting the clinical outcomes among patients with HCC receiving antiangiogenic therapy. The rs699947 C allele, among others, emerged as potential predictive markers of longer progression-free survival and overall survival [16]. However, the previous studies show conflicting results regarding the potential impact of rs699947 on HCC.

This conflict and different data obtained from different studies could be caused by many factors such as difference in ethnicity, the variation in the underlying risk factor and the etiology of liver disease or the small sample size. A previous meta-analysis was performed and showed a significant association between rs699947 and HCC in the overall population (only recessive comparison). The same meta-analysis showed no significance in the East Asian population. So, the authors concluded that this polymorphism could confer risk in certain ethnicities [17]. This encouraged us to do more investigations on this topic enrolling a different population.

Interestingly, the current study demonstrated that the AA and the AC genotypes, in addition to A-allele frequency, were lower in HCC compared with both the cirrhotic and control groups. Liu and his colleagues found similar results but their study enrolled only HCC and control group. Thus they lack the data about cirrhotic patients which gives a better view as cirrhosis is the major risk factor for HCC development [18].

Another study was done in Indonesia and enrolled patients with liver cirrhosis and chronic liver disease in addition to HCC and control groups. Their study found a significant association at the allelic level only [19]. The present study showed that patients with HCC had the least frequent A-allele followed by the control while the cirrhotic group showed the highest A-allele frequency. On the other hand, the Indonesian study showed that the A-allele was least frequent in the cirrhotic group followed by the HCC then the chronic liver disease group and the highest frequency was found in the control group [19].

In the current study, the CC genotype was the most frequent in HCC patients (56.2%), followed by the CA (31.2%) and the least frequent was the AA (12.6%). Similarly, the distribution of genotypes, among patients with HCC, based on Kong et al. [20] and Wu et al. [21] studies revealed that CC genotype (52.9%; 52.2%) is the most frequent compared with CA (39.9%; 43.5%) and AA genotypes (7.2%; 4.3%). Genotype and allele prevalence in addition to the site of polymorphisms of VEGF are still in doubt and further studies are needed to study this point.

It is to be noted that the current study was performed on a different ethnic population, Egyptians, and the main etiological factor was HCV infection rather than HBV infection which was the main etiology

in the previous studies. This could, at least partly, provide an explanation for the inconsistencies in results.

Based on the presence of rs699947 in the promotor region at −2578, it was expected to influence the expression of VEGF. However, controversial results were reported regarding the functional activity of rs699947. One of these studies claimed that the C/C genotype was associated with higher VEGF serum levels compared with the C/A genotype [22]. Another study found that the A/A genotype was associated with a higher VEGF level [23]. On the other hand, no relation between the genotype and the serum VEGF levels was also reported among other studies [18, 24]. Regarding the association between rs699947 and disease activity, there was an association with total bilirubin and serum albumin which are the two constituents of the Child Pugh scoring system. However, this score itself was not influenced by rs699947.

CONCLUSION

It could be concluded that the presence of VEGF rs699947 A-allele confers a lower risk for HCC development especially in Egyptian patients with preexisting HCV infection.

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