Research Article

Involvement of vascular endothelial growth factor (VEGF) gene polymorphism in hepatocellular carcinoma of HCV patients from local population

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Abstract

Development of Hepatocellular carcinoma (HCC) after Hepatitis C virus (HCV) infection is common cause of poor long-term survival. This study aimed to evaluate the effect of Vascular Endothelial growth factor (VEGF) genes polymorphism as a risk factor of Hepatocellular carcinoma (HCC) in chronic hepatitis C virus in Pakistani population. VEGF regulates angiogenesis so it is mainly involved in progression and development of solid tumor. The results showed that HCC patients had a higher frequency of the VEGF-634 GG genotype. In this study results revealed that VEGF Genotype GG is not associated with the risk of HCC in HCV infected patients in Punjab region of Pakistan.

Keywords: HCV; HCC; VEGF Polymorphism

Introduction

Patients with chronic HCV infection progress to liver cirrhosis and ultimately to the stage of hepatocellular carcinoma (HCC). HCC is leading cancer which develop more in men worldwide [1]. HCC is a global health problem, it is the fifth most

common cancer and third leading cause of cancer related deaths worldwide [2]. 10 million of population of Pakistan has been infected by HCV [3] and its sero frequency is high 0.4 [4]. About ~2–3 % of world population has been infected whom around 85 % will lead to chronic infection with

most dangerous outcomes i.e., liver fibrosis, cirrhosis and ultimately hepatocellular carcinoma (HCC) [5].

Different members of cytokines play a key role in the progression of HCC. VEGF plays a vital role in tumor angiogenesis by development of new blood vessels which resulted in invasion and metastasis which indicated that VEGF is potent biomarker [6]. A balance is established between cell growth and cell death; vascularization is important for further proliferation and spread of the tumor [7, 8]. Early tumor growth and metastasis is attenuated more frequently occurs upon VEGF inhibition, suggesting a vital role in carcinogenesis [8, 9]. VEGF that was initially thought to be expressed predominately in endothelial cells indicates poor prognosis in HCC [10, 11]. Knockdown of VEGF showed a reduced proliferation, survival and migration in hepatoma cell lines which confirms the role of VEGF in HCC progression [10]. Genetic variation induces in VEGF gene expression that induced in angiogenesis and HCC in HCV patients [12].

Different polymorphism reported in VEGF is associated with risk to HCV induced hepatocellular carcinoma in Pakistani patients.

Materials and methods

A total 288 patients were recruited in this study and they were classified into 3 groups. Group III (96 patients of HCC having HCV infection), Group II (98 patients of HCV infection) and Group I comprises of 98 healthy objects of different ages and sex without HCV infection. The confirmation of HCC was done by using different techniques i.e., magnetic resonance imaging and abdominal US. The different parameters of HCC patients were obtained i.e., tumor size and metastasis. All patients were recruited from different hospitals of Punjab. The protocol was approved by the Ethical Committee of Government College

University, Faisalabad. Written consent was also obtained from each case.

Sample collection

Blood samples were drawn from all the subjects. Serum were separated immediately and stored at -20°C. Liver functioning tests i.e., Bilirubin, Aspartate aminotransferase (ALT), Alanine aminotransferase (ALT), total protein and albumin were measured from serum by routine enzymatic methods. Alpha fetoprotein was also measured by ELISA method. Genomic DNA was extracted from EDTA-anticoagulated whole blood using a spin column method to the manufacturer protocol (QIA amp Blood Kit, Qiagen). At -20 C, DNA was stored for further use.

Polymorphism analysis of VEGF

Polymorphism of VEGF -634 promotor was done with the help of polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method described by Carturan *et al.* [13] for VEGF “5-GTAGCAAGAGCTCCAGAGAGAAAGT-3” and “5-TGGACGAAAAGTTTCAGTGCGACG-3”, as forward and reverse primers respectively [13]. Total volume (25µl) of PCR reaction mixture contain 10 µg genomic DNA, 5 pmol of each primer, 2X PCR mix having (200µmol/l of each dNTP, 5 µl of 10X reaction buffer, 1.25 U Taq Gold Polymerase and 4 mmol/l MgCl₂ .This PCR reaction mixture was initially denatured at 95°C for 10 min, followed by 35 cycles of 94 °C for 30 s, 58°C for 30 s, 72°C for 45 s, and final extension cycle 72 °C for 10 min. the PCR product were overnight digested with with appropriate restriction enzyme BsmFI for VEGF (New England Bio labs, Beverly, MA, USA) and separated by agarose gel electrophoresis.

Statistical analysis

The continuous variables were expressed as mean ± SD. Differences in the frequency of polymorphisms were calculated the chi-squared test. A p-value <0.05 was used to assess significance.

Results

General characteristics of the subjects

A total 288 patients were selected and they were categorized into 3 groups. Group I comprises of 98 healthy objects, Group II (96 patients of HCV infection and Group III (94 patients of HCC having HCV infection. Characteristics of these subjects are summarized in Table 1

No significant differences were observed in age and gender distribution between the

cases and control (p value were 0.021 and 0.000 respectively). The smoking rates of the 2 groups were also nearby (p=0.987), However the drinking status is also non-significant (p=0.550).AST, Alpha fetoprotein and total bilirubin levels were considerably higher among the cases compared to controls (p > 0.05). However ALT, total protein and albumin were considerably lower among cases compared to controls (p < 0.05).

Table 1.

Variables		Group I N= 98	Group II N= 96	Group III N=94	P Value
Gender	Male	68 (69.38%)	70 (72.91%)	80 (85.50%)	0.021
	Female	30 (30.61%)	26 (27.08%)	14 (14.89%)	
Age		47.49 ±2.61	50.15 ± 2.36	52.99 ± 3.99	0.000
Smoking status		11 (11.70%)	14 (14.58%)	12 (12.76%)	0.987
Drinking status		5 (5.10%)	6 (6.25%)	6 (6.38%)	0.550
ALT (U/L)		22.71 ± 5.22	65.33 ± 31.52	64.99 ± 39.47	*0.000 # 0.947 **0.000
AST (U/L)		32.24 ± 7.54	72.79 ± 31.73	111.09 ± 70.71	*0.000 #0.000 **0.000
Albumin (g/dL)		4.6 ± 0.28	3.6 ± 0.49	3.2 ± 0.65	*0.000 #0.000 **0.000
Total proteins (g/dL)		7.46 ±0.43	7.09 ± 0.41	5.83 ± 0.83	*0.000 #0.000 **0.000
Total bilirubin (μM)		0.5 ± 0.87	1.46 ± 0.56	2.03 ± 0.75	*0.000 #0.000 **0.000
AFP (μg/L)		4.03 ± 1.95	30.71 ± 27.83	412.35 ± 285.23	*0.000 #0.001 **0.000

n=number, %=percentage, p≤0.05=statistically significant

*= Comparing between Control group and Chronic HCV, #= Comparing between HCV and HCC group, **= Comparing between HCC and Control group

Clinical characteristics of patients with HCC

Clinical characteristics of HCC patients, 24 patients (25.53%) had portal vein

thrombosis and 10 patients (10.63%) had lymph node metastasis as mentioned in Table 2.

Table 2. Clinical characteristics of patients with HCC

Metastasis	
Present	10 (10.63%)
Absent	84 (89.36%)
Portal Vein thrombosis	
Positive	24 (25.53%)
Negative	70 (74.46%)
Tumor Size	
<20 mm	48 (51.06%)
21-40 mm	38 (40.42%)
≥ 40 mm	8(8.52%)

Association between VEGF gene polymorphism 634G>C and HCC

Our study concluded that -634G/C polymorphism was involved in development of Hepatocellular carcinoma Table 3. Hardy-Weinberg equilibrium was found in all the studied groups. -Weinberg equilibrium in each studies group. The genotype GC -634 in VEGF gene was presented nearly equal in all the subjects (HCC group has 29.78%, HCV patients without HCC, has 23.95% and

healthy controls, have 20.59%. HCC patients showed a lower occurrence of the CC genotype of the VEGF-634 polymorphism and a higher occurrence of the GG genotype as healthy controls. The incidence of HCC was higher among HCC group carrying the CC genotype than in patients having the GG genotype was not related to any genotype. [OR (95%CI) =0.962 (0.476-1.956), P = 0.008].

Table 3. VEGF genotypes in HCC, HCV and control group

		Healthy Control N=98 n (%)	HCV Patients N=96 n (%)	OR (95% CI)	P-value	HCC Patients N=94 n (%)	OR (95%CI)	P-value
VEGF -634	GG	49 (50)	53 (55.20)	1(Reference)	---	47 (50)	1(Reference)	---
	GC	29(29.59)	23 (23.95)	1.334 (0.704-2.526)	0.375	28 (29.78)	0.742 (0.389-1.414) 1.009 (0.543-1.875)	0.3641 0.9762
	CC	20 (20.40)	20 (20.83)	0.974 (0.486-1.953)	0.941	19 (20.21)	1.038 (0.513-2.100) 0.962 (0.476-1.946)	0.9151 0.008
	G allele	127	129	1(Reference)	---	122	1(Reference)	---
	C allele	69	63	1.112 (0.730-1.693)	0.619	66	0.902 (0.590-1.380) 0.995 (0.654-1.514)	0.6361 0.9842

¹= Healthy Vs HCC

²=HCV Vs HCC OR= Odd ratio,

CI=Confidence Interval

<0.005 Significant

Discussion

Hepatocellular carcinoma is fifth most abundant diagnosed cancer globally [14]. Chronic liver disease more commonly affected the life in Africa and Asia. HCV patient with or out cirrhosis may develop

hepatocytes (continuous regeneration and inflammation) which is the main cause of development of hepatocellular carcinoma. Comparing to other tumor category, inflammation and cirrhosis in HCV patients makes it more difficult to diagnose in early

stages [15]. Early diagnosis of liver carcinoma makes it possible for the patients to get the most optimal and beneficial therapy available. Firstly serum tumor markers have been used for diagnosis of malignant tumors [16, 17] they could also support to computed tomography and ultrasonography in diagnosis of HCC.

Development of liver cirrhosis in HCV infection may lead towards mortality and morbidity. The long term hepatic infection brought changes from minimum to extensive fibrosis, cirrhosis and HCC. In HCV infection continuous inflammation firstly but slowly result in liver fibrosis and then towards HCC. Increased cytokines production has been noticed in fibrosis due to viral infection and uncontrolled activation of the immune system [18]. Numerous genes and their allelic variation have been analyzed as potent biomarker affecting HCV [19].

In this study single nucleotide polymorphism was investigated for VEGF and its vulnerability to HCC in Pakistani population who already infected by HCV. The GG genotype was the most common genotype amongst all subjects relative to the CC and GC genotypes. When compared with the two previous studies of HCC, we observed significant differences in the genotypic and allelic frequencies of SNP rs2010963 (-634) amongst HCC patients. In the present study, the prevalence of the genotype CC was lower than that of GC or GG, and the G allele was more common than allele C [20, 21]. The two previous studies also reported the predominance of the GC genotype, but the difference in prevalence was less striking than the outcomes of this study. A previous study revealed that the genotype CC may predict an increased survival of HCC patients after surgery and chemotherapy [20]. The prevalence of the CC genotype in this study was lower than the other genotypes (HCC 1

pts, HCV 3 pts, and healthy 2 pts). The prevalence of the VEGF SNP rs2010963 (-634) in HCV subjects has not been examined previously. Presently GG genotype and G allele were most common amongst all subjects. The frequency of the GC genotype and G allele in healthy subjects was significantly higher than in HCC and HCV subjects. The GG genotypes in HCV subjects are higher than healthy HCC which indicates that the increased frequency of the GG genotype reflects a decreased activity/expression of the VEGF protein. Polymorphism of the VEGF gene located in the promoter may increase the activity/expression of VEGF gene protein [22-24].

The frequency of the GC genotype in HCV subjects are lower than healthy subjects, indicating that neo-angiogenic activity may have been suppressed relative to HCC and chronic active hepatitis. Furthermore, the number of hepatocytes in HCV is relatively small because most hepatocytes are replaced by fibrotic tissue. Mediators of pro-angiogenesis activity placental growth factor (PIGF)) and (VEGF, platelet-derived growth factor (PDGF) are required in cirrhosis to maintain splanchnic hyperdynamic circulation during portal hypertension [25].

Kong *et al.* [20] revealed that there is no difference in the type of VEGF SNP genotypes amongst HCC subjects (including SNP VEGF -634) with respect to clinical condition, despite the differences in size and tumor invasion. Furthermore, the presence of the CC genotype may predict survival and the GG genotype may be associated with mortality in HCC subjects. The role of SNP VEGF -634 in predicting survival remains debatable, and only few studies have been conducted on Liver cirrhosis and chronic hepatitis subjects. Previous studies on HCC patients examined the association of VEGF SNPs with survival and recurrence of HCC after surgery, transplantation, and

chemotherapy, as well as SNP VEGF 634 as a predictor of survival [20, 21].

However, a meta-analysis revealed that SNP VEGF -634 G/C did not indicate the risk of malignancy on malignant solid tumors [11]. This research study involved only hospital-based subjects who were recruited with the cross-sectional method, which cannot prove causality (cause–effect relationship) and cannot represent real situations in the general population. The role of other VEGF SNPs in VEGF expression in liver tissue and the levels of VEGF in the blood remain unclear. It will be important to evaluate polymorphisms in VEGFR not examined in this study. Biopsy is an invasive procedure that was often avoided by our subjects, and surgery (hepatoectomy) and transplantation were rarely performed in our hospitals thus, the researchers were unable to obtain a sample of hepatic tissue to measure the expression level of VEGF, VEGFR, and micro vascular density (MVD) in hepatic tissue.

Conclusion

This study concluded that VEGF polymorphism (VEGF-634) is not associated with cirrhosis, HCC and survival. However there is a relationship between genotype GG is persistently raised in HCC and HCV in patients as compared to normal. For identification of high risks for HCV patients genetic testing may be a good tool.

Authors' contributions

Conceived and designed the experiments: S Jahan, Performed the Experiments: MU Ghani, A Waheed & MS Iqbal, Analyzed the Data: MS Masoud & UA Ashfaq, Contributed reagents/ materials/ analysis tools: A Haque, S Khaliq, N Afzal & M Qasim, Wrote the paper: F Saeed, MU Ghani & A Waheed.

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