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Original article

RECK gene polymorphisms in hepatitis B-related hepatocellular carcinoma: A case-control study

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ABSTRACT

Background and study aims: Chronic hepatitis B (CHB) infection is a major risk factor for hepatocellular carcinoma (HCC). The RECK gene is a critical tumor suppressor gene. This study aimed to assess the association between RECK gene single nucleotide polymorphisms (SNPs) and the development of HCC in Egyptian patients with chronic hepatitis B.

Patients and method: In this case-control study, we enrolled patients with CHB from the Gastroenterology Department, Benha University, from June 2016 to February 2018. The RECK gene SNP rs10814325 was identified using real-time PCR allelic discrimination via TaqMan SNP genotyping assays (Applied Biosystems, USA).

Results: We enrolled 140 participants in this study. The participants were divided into Group I, which comprised 50 participants with CHB only, Group II, which comprised 50 participants with CHB and HCC, and Group III, which comprised 40 healthy participants. A significantly higher hepatitis B virus DNA viremia level was found in patients with HCC. The predominant RECK genotype was the T/T allele, followed by the T/C allele; however, no significant difference in the distribution of RECK gene SNPs was found between the study groups. No statistically significant difference in RECK gene SNPs was reported among patients with HCC of different Child classes or based on the number, site, size of HCC, and lymph node involvement. Receiver operating characteristic curves showed that a serum alpha-fetoprotein level of 92 ng/ml was 96 % sensitive and 100 % specific for the detection of HCC, with an area under the operating characteristic curve of 0.98.

Conclusion: RECK gene SNPs have no significant association with the development and characteristics of hepatitis B-related HCC in Egyptian patients.

Introduction

Hepatocellular carcinoma (HCC) is the sixth most common cancer worldwide and the fourth most common cancer in Egypt where chronic hepatitis C is the most common underlying etiology. Worldwide, HCC is the fourth most common cause of cancer-related mortality. In Egypt, it is the most common cause of cancer-related morbidity and mortality [1]. Hepatic carcinogenesis is a complex process with multiple underlying factors and pathways. Both personal genetic and environmental factors influence the evolution of HCC. Thus, the occurrence of HCC varies across patients [2]. Understanding these biological factors may help identify patients who are most likely to benefit from particular preventative strategies or screening policies. Numerous genetic analyzes have highlighted the association between single nucleotide polymorphisms (SNPs) as an underlying factor of HCC [2–6].

Chronic hepatitis B virus (HBV) infection is responsible for over 50 % of HCC cases around the world [7]. In Egypt, the incidence of chronic HBV infection decreased significantly in the general population following the introduction of universal HBV vaccination for infants in 1992 [8]. The rates of HBV surface antigen and HBV core total antibody positivity among those aged 15–59 years in Egypt are 1.5 % and 15.7 %, respectively [9]. HBV genotype D is the most common genotype among

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Egyptians; however, genotype E caused a breakthrough infection after the national HBV vaccination campaign in Egypt [10].

The development of HCC in patients with chronic HBV infection is related to differences in the HBV genotype, genetic variations, tumor-specific somatic mutations, and environmental factors. The identification of viral and host variations can improve the rate of early diagnosis and prognosis whereas the identification of somatic mutations promoting hepato-carcinogenesis can guide precision therapy for patients with HCC. Ten genotypes of HBV (A to J) were identified, with genotype D being associated with a higher risk of HCC. The risk of HCC is similar in genotypes A and D [11].

HBV infection causes HCC when the viral proteins disturb the hepatocyte signaling pathways and, thus, affect gene expression and function, or when HBV DNA integrates the host genome to induce chromosomal instability and chronic inflammation that alters specific signaling pathways [11]. The reversion-inducing-cysteine-rich protein with Kazal motifs (RECK) is a cell membrane-anchored type of glycoprotein that may act as a metastasis suppressor against activated Ras oncogenes [12]. It is widely expressed in normal tissues [13]. In animal models, RECK expression inhibits tumor invasion, metastasis, and angiogenesis. High RECK expression in tumor tissues also is associated with better survival [14]. Thus, the upregulation of RECK may be a promising approach for treating malignant tumors [15]**.** This study aimed to identify possible associations between the RECK gene polymorphism rs10814325 and the risk of developing HCC among Egyptian patients with chronic HBV.

Patients and methods

In this case-control study, we included 140 participants from the Department of Hepatology, Gastroenterology, and Infectious Diseases at Benha University Hospitals. They were recruited from June 2016 to February 2018. The ethical committee of Benha University approved the study, which was conducted per the ethical guidelines of the declaration of Helsinki. All participants gave their written informed consent before their enrolment in the study.

The participants were divided into three groups (Groups I, II, and III). Group I (the chronic HBV group) included 50 patients with chronic HBV not complicated by HCC who had persistent HBV surface antigen positivity for more than six months and the presence of HBV DNA in serum identified via quantitative PCR. Group II (the HCC group) included 50 patients with chronic HBV and HCC, which were diagnosed according to the American Association for the Study of Liver Diseases guidelines using triphasic CT, MRI, serum alpha-fetoprotein (AFP) levels, or a combination of the above modalities. Group III (the control group) included 40 healthy participants with negative viral markers for the HBV surface antigen, HBc total, HBc IgM, and the hepatitis C antibody.

We included people older than 18 years with chronic HBV, with or without HCC. All included participants were naïve to antiviral therapy. Our exclusion criteria were pregnancy, hepatitis C Ab + status, chronic liver diseases of causes other than chronic HBV infection, malignancies other than HCC, or refusal to give one's informed consent.

All participants underwent thorough history-taking and complete clinical examinations. Baseline laboratory investigations included complete blood cell counts (Sysmex, USA), serum ALT (U/l), serum AST (U/l), serum albumin (g/ dl), serum total bilirubin level (mg/dl) were performed by Biosystem A15 auto-analyzer (Barcheluna, spain). PT by (CoADATA 504 Germany). HBV surface antigen, HBV envelope antigen, HBV core antibodies total, HBV envelope antibodies, and the hepatitis C virus antibody were done via enzyme-linked immunosorbent assay (ELISA) using Human ELISA kits supplied by Adaltis (Freiburg, Germany). HBV DNA was extracted from whole blood samples using a QiaAmp DSP virus kit, followed by amplification using the Artus HBV PCR kit (Qiagen, Germany) and the Step One Plus PCR system (Applied Biosystems, USA) to detect the viral load. The serum AFP level was assessed using Human AFP ELISA kits (Glory Science, USA). Abdominal ultrasonography and triphasic CT of the abdomen and pelvis were conducted in Group II (HCC group) to confirm and characterize the staging of HCC.

Identification of the RECK gene polymorphism rs10814325

Three milliliters of peripheral blood were collected from each subject, under aseptic conditions, into EDTA-containing vacutainers. Whole blood samples were used for DNA extraction using PureLink® Genomic DNA Kits for the purification of genomic DNA (Life Technologies, USA), following the manufacturer's protocol. A concentration of 1 μg from each sample was used for genotyping via the qPCR assay on a Nanodrop One spectrophotometer (Thermo Scientific, USA).

Genotyping of RECK gene polymorphism rs10814325 was performed using a TaqMan Predesigned SNP Genotyping Assay (Applied Biosystems, USA) and TaqMan™ Genotyping Master Mix (Applied Biosystems, USA) per the manufacturer's instructions. Genotyping assays were performed and analyzed using a StepOne plus real-time PCR system (Applied Biosystems, USA) under the following reaction conditions: 95 °C for 10 min, followed by cycling for 40 cycles of denaturation at 92 °C for 15 *sec* and annealing and extension at 60 °C for 1 min. No-transcript controls were used for each PCR run, with primer sets for each allele. Allelic discrimination analysis was performed using Step One plus to calculate normalized dye fluorescence (ΔRn) for allele T (wild-type) or allele C (mutant). The software program identifies allele T (homozygous T/T), allele C (homozygous C/C), or heterozygous (T/C).

Statistical analysis

The collected data were tabulated and analyzed using SPSS version 16 (SPSS Inc, USA). Categorical data are presented as frequencies and percentages while quantitative data are expressed as either the mean ± standard deviation or the median and interquartile range for variables with normal and skewed data distributions, respectively. The chi-square test (X^2) or Fisher's exact test was used to analyze categorical variables. The odds ratio and corresponding 95 % confidence interval were calculated when accessible. Quantitative data were tested for normality using the Shapiro–Wilks test, assuming normality at $P > 0.05$. Student's *t*-test was used to compare normally distributed variables between two independent groups. Variables with skewed data distributions were analyzed using the Mann–Whitney *U* test for two independent groups. The difference between three independent means was analyzed using the ANOVA test for normally distributed variables or the Kruskal–Wallis test for variables with skewed data distributions. In the case of statistical significance, the Kruskal–Wallis test was followed by pairwise comparisons using the Bonferroni test to detect the significant pairs. A receiver operating characteristic (ROC) curve analysis was performed to assess the validity of the RECK gene genotype and AFP for predicting HCC. $P < 0.05$ was considered statistically significant.

Results

Table 1 summarizes the demographic and laboratory characteristics of the study groups**.** Participants with HCC were significantly older than those in the other two groups. No significant gender difference was observed between groups. The amount of HBV DNA was significantly higher in the HCC group than in the chronic HBV group. Table 2 summarizes the tumor characteristics in the HCC group. Most HCC lesions occurred in the right lobe. The mean size of HCC lesions was 4.8 ± 3.2 cm, and no portal vein thrombosis was observed in the included patients. Lymph node metastasis was detected in 16 (32 %) patients.

Table 3 shows the distributions of RECK genotypes and alleles among the study groups. No statistically significant difference in the distribution of RECK genotypes and alleles was found between the three groups. Among patients with HCC, no statistically significant differences were observed based on different RECK gene polymorphisms with respect to jaundice, ascites, encephalopathy, history of hematemesis, melena,

Table 1
Demographic and Laboratory features of the study groups.

Variable	CHB group	HCC group	Control group	P-value	Significant Pairs
Age in years	39.6 ± 11.4	52.6 ± 15.9	42.5 ± 6.2	**0.001**	
Gender					
Male/female (%)	16/9 (64/36 %)	17/8 (68/32 %)	13/7 (65/35 %)	**0.95**	
Hemoglobin (gm/dl)	12.6 ± 1.99	11.1 ± 2.77	13.1 ± 0.89	0.012	**GII ≠ GIII**
Total leucocytic count (×103)	5.61 ± 1.97	5.96 ± 3.55	6.35 ± 1.6	0.41	———
Platelet count (×103)	209.3 ± 112.21	144.7 ± 102.77	387.1 ± 77.1	<0.001	**GII ≠ GI** **GII ≠ GIII** **GI ≠ GIII**
ALT (u/l)	50.8 ± 49.7	72.6 ± 45.2	24.8 ± 9.1	<0.001	**GII ≠ GIII** **GI ≠ GIII**
AST (u/l)	44.2 ± 27.5	84.8 ± 73.0	23.5 ± 7.49	<0.001	**GII ≠ GIII** **GII ≠ GI**
INR	1.04 ± 0.16	1.22 ± 0.32	0.97 ± 0.12	0.041	**GII ≠ GI** **GII ≠ GIII**
Serum Albumin (g/dl)	3.79 ± 0.62	3.19 ± 0.49	3.95 ± 0.49	<0.001	**GII ≠ GI** **GII ≠ GIII**
Total Bilirubin (mg/dl)	1.04 ± 0.30	1.96 ± 0.94	0.48 ± 0.21	<0.001	**GII ≠ GI** **GII ≠ GIII** **GI ≠ GIII**
CHILD-Pugh score					
A	20 (80.0 %)	11 (44.0 %)			
B	5 (20.0 %)	4 (16.0 %)	———	0.001	
C	0	10 (40.0 %)			
Alpha-fetoprotein (ng/ml)	4.85 ± 4.1	5956.9 ± 15766.0	2.33 ± 2.08	<0.001	**GII ≠ GIII** **GII ≠ GI**
HBV DNA (IU/ml)	58 821.7	284 115.4	———	0.007	

ALT: alanine transaminase, AST: Aspartate transaminase, INR: International normalized ration, G: Group.

Table 2
HCC tumor characteristics.

Focal lesion		Number (Total = 50)	Percent
Number	≤2	36	72.0
	>2	14	28.0
Site	Right lobe	34	68.0
	left lobe	4	8.0
	Both lobes	12	24.0
Size (cm)	<5	34	68.0
	≥5	16	32.0
Lymph node metastasis	No	34	68.0
	Yes	16	32.0

Table 3
Distribution of RECK genotypes and alleles among the studied groups.

RECK Genotype			Group I (Non-HCC) N = 50	Group II (HCC) N = 50	Group III (Control) N = 40	P-value
Genotype	**TT**	**Number**	**30**	**34**	**20**	**0.78**
		% within Group	60.0 %	68.0 %	50.0 %	
	TC	**Number**	**16**	**14**	**16**	
		% within Group	32.0 %	28.0 %	40.0 %	
	CC	**Number**	**4**	**2**	**4**	
		% within Group	8.0 %	4.0 %	10.0 %	
Allele	**T**	**Number**	**76**	**82**	**56**	**0.41**
		% within Group	76.0 %	82.0 %	70.0 %	
	C	**Number**	**24**	**18**	**24**	
		% within Group	24.0 %	18.0 %	30.0 %	

Child–Pugh classes (P = 0.53), baseline laboratory results, or HBV DNA viral load **(**Table 4**).** Patients with HCC and the RECK TT genotype had significantly lower hemoglobin levels (10.2 ± 2.33 g/dl), than those with the CC or TC genotypes (13.1 ± 2.76 g/dl). Among patients with

Table 4
Clinical signs and lab results and RECK gene polymorphisms distribution in the HCC group.

Variable	TT (n = 34)		TC/CC (n = 16)		P-value
	No.	%	%	No.	
Jaundice	No	12	35.3	8	0.67
	Yes	22	64.7	8	
Ascites	No	20	58.0	6	1.00
	Yes	14	41.2	10	
Encephalopathy	No	20	58.8	10	1.0
	Yes	14	41.2	6	
History of Hematemesis	No	16	47.1	12	0.23
	Yes	18	52.9	4	
History of Melena	No	18	52.9	12	0.40
	Yes	16	47.1	4	
Child-Pugh class A	12 (35.3 %)		**10 (**62.5 %)		
Child-Pugh class B	6 (17.6 %)		**2 (**12.5 %**)**		
Child-Pugh class C	16 (47.1 %)		**4 (**25.0 %**)**		
Hb% (g/dl)	10.2 ± 2.33		13.1 ± 2.76		**0.014**
TLC ($\times 10^3$)	6.33 ± 3.92		5.18 ± 2.66		0.66
PLTs ($\times 10^3$)	132.3 ± 100.8		171.1 ± 108.5		0.28
ALT (u/l)	77.1 ± 51.0		63.1 ± 30.3		0.77
AST (u/l)	92.2 ± 85.4		69.1 ± 34.5		0.66
AFP (ng/ml)	7784.2 ± 18840.09		2073.8 ± 3946.89		0.32
HBV, DNA (IU/L)	351545 ± 1350674		140828 ± 365578		0.77

HCC, no statistically significant difference in the number, site, size, or lymph node metastasis was observed between RECK genotype distributions **(**Table 5**).** As shown in Fig. 1, according to the ROC curve, AFP at a cut-off of 92 ng/ml showed 96 % sensitivity, 100 % specificity, 100 % positive predictive value, and 97.8 % negative predictive value with an accuracy of 98.5 % and an area under the curve of 0.98 (95 % confidence interval 0.96–1.0).

Discussion

To our knowledge, this is the first study to examine the relationship between RECK gene SNPs and the development of HCC in patients with chronic HBV. Regarding the frequency of distribution of RECK genotypes and alleles among the different groups, there were no significant

Table 5
Focal lesion characters among HCC group according to genotype frequency.

Variable		TT (n = 34) No. (%)	TC/CC (n = 16) No. (%)	OR (95 % CI)	P value
Number	≤2	26 (76.5 %)	10 (62.5 %)	1.95 (0.31–12.0)	0.64
	>2	8 (23.5 %)	6 (37.5 %)		
Site	Rt. lobe	26 (76.5 %)	8 (50.0 %)	———	0.29
	Lt. lobe	2 (5.9 %)	2 (12.5 %)		
	Both	6 (17.6 %)	6 (37.5 %)		
Single FL Size (cm)	<5	24 (70.6 %)	10 (62.5 %)	1.44 (0.25–8.5)	1.0
	≥5	10 (29.4 %)	6 (37.5 %)		
Density	Hypodense	30 (88.2 %)	14 (87.5 %)	1.07 (0.08–13.9)	1.0
	Hyperdense	4 (11.8 %)	2 (12.5 %)		
Lymph node involvement	No	22 (64.7 %)	12 (75.0 %)	0.61 (0.9–4.0)	1.0
	Yes	12 (35.3 %)	4 (25.0 %)		

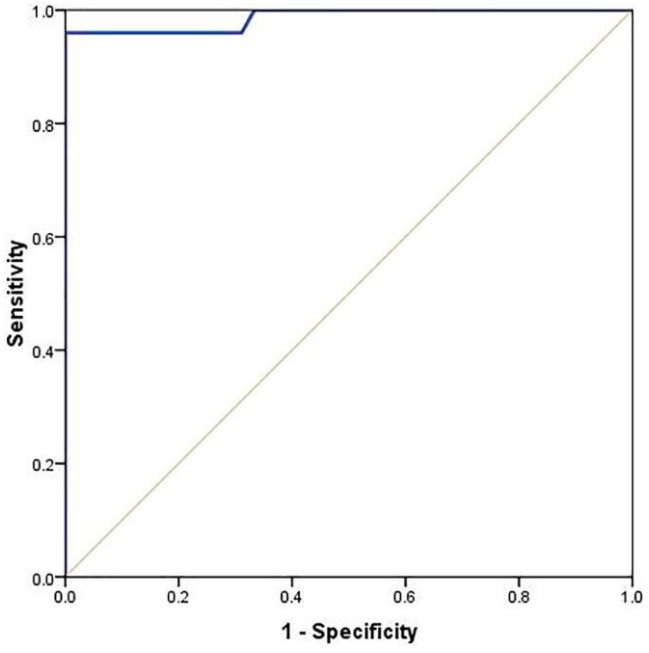

Fig. 1. Receiver operating characteristic curve for the diagnostic performance of alpha-fetoprotein in the hepatocellular carcinoma group.

differences between the study groups. HBV infection is considered a major risk factor for liver cirrhosis and HCC. HBV infection becomes chronic in about 10 % of infected patients and increases the risk of HCC evolution approximately 100-fold compared to the normal population [16].

The RECK gene is a negative target for oncogenic signals. RECK gene downregulation is a precursor for malignant transformation. RECK mRNA is expressed in most normal human tissues; however, its expression is lower in HCC tissues than in surrounding non-malignant tissues. Thus, patients with HCC with higher RECK mRNA expression tend to have lower grades of invasion, lower rates of metastasis, better survival rates, and better prognoses. These findings indicate that the RECK gene is a principal tumor suppressor gene and emphasize the feasibility of the use of RECK mRNA as a promising prognostic molecular marker for HCC [17].

SNPs are genes that encode cancer susceptibility factors. SNPs have been documented to influence gene expression, protein function, and disease susceptibility in certain individuals. Of the 13 SNPs documented in the RECK gene, four are present in the gene's coding sequences (exons 1, 9, 13, and 15) and the remaining nine occur in the intervening sequences of the gene in the vicinity of the exon/intron boundaries [14]. The present study aimed to assess the relationship between RECK SNP rs10814325 and the development of HCC in Egyptian patients with chronic HBV infection. Previous research has confirmed the relationships of RECK expression and gene status with tumor metastasis and prognosis, showing that promoter hypermethylation silencing of RECK mRNA is associated with poor survival in HCC [18] and shorter survival in patients with invasive breast cancer [19]. In patients with oral cancer, those who had the RECK polymorphism had a higher incidence of malignant involvement of the lymph nodes of the neck than wild-type carriers [20]. Low RECK expression among patients with colorectal cancer [21], esophageal cancer [22], and non-small cell lung cancer [23] is associated with a higher incidence of malignant lymph node involvement. Badawy et al. [24] studied cirrhosis and HCC susceptibility in patients with chronic hepatitis C and reported that the RECK promoter rs16932912 polymorphism has no significant influence on the occurrence or progression of HCC.

Fakhry and colleagues [25] reported no significant association regarding the frequency distribution of RECK genotypes and rs10814325 SNP, specifically among those with hepatitis C-related liver cirrhosis and hepatitis C-related HCC, compared with controls. In their study, 75 % of patients with HCC complicated by lymph node metastasis had the TT genotype. Abou-Elela and colleagues [26] also used PCR restriction fragment length polymorphism to detect RECK gene polymorphisms rs11788747 and rs16932912 in patients with hepatitis C-related HCC or liver cirrhosis and a control group. They found that RECK rs11788747 was potentially involved in HCC pathogenesis but rs16932912 was not.

The RECK SNP homozygous mutation rs1081432 has been studied in individuals without chronic HBV, revealing a 2.68-fold risk of HCC (95 % confidence interval 1.35–5.34); however, heterozygosity was not statistically significant [27]. An ethnicity-related variation in the frequency of RECK gene rs10814325 polymorphisms has been documented, which could help explain the contradictory findings for RECK polymorphisms in different populations [15].

Comparing the laboratory and clinical findings of our patients and the genotype frequency distribution in the HCC group, we found a significant difference in the hemoglobin level between genotypes TT and genotypes TC or CC ($P = 0.014$). However, we found no significant difference in the clinical manifestation, Child–Pugh classification, or triphasic CT criteria of focal lesions between genotype TT and genotype TC or CC. Similar results were obtained by Chung et al. [15], who observed no significant association between gene polymorphisms and clinical-pathologic states of the studied patients with HCC. Similarly, Elattar et al. [28] investigated the potential effect of RECK gene promoter SNPs and hepatitis C-related HCC susceptibility on clinical and laboratory characteristics in Egyptian patients and found no significant association between laboratory parameters and RECK promoter genotypes.

An expected pattern was encountered in the laboratory data of our study. The hemoglobin level, platelet count, prothrombin concentration, total protein, and albumin were significantly lower in both HCC and chronic HBV groups than in the normal controls. However, results for the prothrombin time, international normalized ratio, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, gamma-glutamyl transpeptidase, total and direct bilirubin, blood urea nitrogen, and creatinine were significantly higher in both HCC and chronic HBV groups compared to the control group. The AFP level was significantly higher in HCC patients. As previously stated, even a small elevation in AFP levels is associated with HCC evolution among patients infected with hepatitis C, whereas AFP levels of < 6 ng/ml indicate a low risk of HCC in those patients, regardless of the fibrosis stage [28–29].

According to the ROC curve analysis of the obtained results, at an AFP cut-off of 92 ng/ml, the sensitivity was 96 % and specificity was 100 % with a positive predictive value of 100 % and negative predictive value of 97.8 %. This finding is comparable to that reported by Sarwar et al. [30], who reported the following at an AFP cut-off of 20.85 ng/ml: 72.2 % sensitivity, 86.2 % specificity, 89.9 % positive predictive value, 64.7 % negative predictive value, and 77.4 % overall accuracy. For RECK genotype TT, sensitivity was 68 % and specificity was 44.4 % with a positive predictive value of 40.5 %, negative predictive value of 71.4 %, accuracy of 52.8 %, and area under the curve of 0.56. Nassar and colleagues studied RECK gene rs10814325 in patients with hepatitis C-related HCC and revealed that the mutant genotype was associated with higher susceptibility to HCC [31].

To our knowledge, this is the first study to examine the relationship between RECK gene SNPs and the development of HCC in patients with chronic HBV. The main limitation of this study is the limited number of participants. In conclusion, RECK gene SNPs had no significant association with the development of HBV-related HCC.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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