

## Original Research Article

# RECK Gene Polymorphisms in Hepatocellular Carcinoma and Cirrhotic Patients Related to Hepatitis C Virus

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**Background:** Hepatocellular carcinoma (HCC) is the sixth most common cancer globally. HCC is the third most common cause of cancer mortality. The development of HCC is a multistep and complex process. Multiple environmental risk factors, including chronic hepatitis B virus (HBV) or hepatitis C virus (HCV) infection, cirrhosis, carcinogen exposure, and a variety of genetic factors contribute to hepatocarcinogenesis. The reversion-inducing-cysteine-rich protein with Kazal motifs (RECK) down-regulation has been confirmed in numerous human cancers and is clinically associated with metastasis. This study investigates the potential associations of RECK single-nucleotide polymorphisms (SNPs) with HCC susceptibility and clinicopathologic characteristics in HCV Egyptian patients. **Methodology:** This study was conducted on a total number of 110 participants admitted to National Hepatology & Tropical Medicine Research Institute. The participants of this study were divided into three groups (30 HCV patients, 30 HCC patients and 30 liver cirrhosis (LC) patients) in addition to 20 healthy individuals as control group were analyzed for RECK SNP (rs16932912) genotyping using real-time. **Results:** RECK rs16932912 mutant genotypes GG/AG showed no significant value in HCC compared to wild type ( $P = 0.373$ ). Mutant genotype was higher in liver cirrhosis group than other groups ( $P = 0.001$ ). High significant levels of ALT, AST, AFP, ALB ( $P < 0.01$ ) among the rs16932912 mutant AG/AA genotypes versus wild GG genotype. **Conclusion:** Our study showed that the presence of A/G genotype was associated with prognosis in HCV-HCC and HCV-LC patients.

**Keywords:** RECK gene, HCV, HCC, LC.

## INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most common cancer worldwide (Butt et al., 2013). HCC is the third most common cause of cancer mortality (Feraly et al., 2010). Many studies show the relation between HCV viral infection and development of Hepatocellular carcinoma (HCC) by continued viral replication, accumulation of chromosomal damage and a great failure in immune response to eliminate hepatocyte chromosomal aberrations (Farazi and DePinho, 2006).

Measuring serum  $\alpha$ -fetoprotein (AFP) levels are used as a diagnostic tool with HCC patients, which is invalid with early staged patients, so there is a great need for genetic markers that could be used for its early detection and, consequently, to start a therapeutic procedure as soon as possible. Single nucleotide polymorphisms in a selected candidate genes, for example, insulin-like growth factor (IGF)-2, IGF-2R,

plasminogen activator inhibitor (PAI)-1, and matrix metalloproteinase (MMP) considered to play a role in the development of many types of cancer diseases, one of them is Hepatocellular carcinoma (HCC) (Weng et al., 2010). Extracellular matrix (ECM) macromolecules are important for creating the cellular environments required during the development and morphogenesis of tissues.

Matrix metalloproteinases (MMPs) are a family of Zinc-dependent endopeptidases that collectively are capable of cleaving virtually all ECM substrates, and play an important role in some physiological and pathological processes (Visse and Nagase, 2003). MMPs activity can be inhibited by some natural and artificial inhibitors. RECK, a newly found membrane-anchored regulator of MMPs can inhibit MMPs activity through several mechanisms, including direct inhibition

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of protease activity, regulation of their release from the cell and possibly through sequestration of MMPs at the cell surface as suggested by many studies (Welm et al., 2002).

RECK inhibits the activity of at least three MMP members, including MMP-2, MMP-9, and MT1-MMP. The reversion inducing-cysteine-rich protein with kazal motifs (RECK), is down regulated when the cells undergo a process of malignant transformation including pancreatic cancer, breast cancer, lung cancer, colorectal cancer, cholangiocarcinoma, gastric cancer, prostate cancer, oral cancer, esophageal cancer and osteosarcoma, which is currently the subject of considerable research activity because of its specific structure and function (Furumoto et al., 2001, Masui et al., 2003, Lei et al., 2007, Takenaka et al., 2004, Takeuchi et al., 2004, Song et al., 2006, Rabien et al., 2007, Takemoto et al., 2007, Long et al., 2008, Qi and Li 2010, Xu et al., 2010, Chung et al., 2011).

A high Reversion-inducing-cysteine-rich protein with Kazal motifs (RECK) mRNA expression - has been considered as a tumor suppressor gene in HCC tumor tissues from patients with better survival and less invasive clinicopathologic features (Zavras et al., 2011). A study in Taiwan shows that only one single nucleotide polymorphism could develop Hepatocellular carcinoma within HCV patients, this SNP had been studied within Reversion-inducing-cysteine-rich protein with Kazal motifs (RECK) gene as its function of Metalloproteinase inhibitor, which is down regulated when the cell undergo a process of malignancy (Yang et al., 2012). Up regulation of RECK could be a valuable therapeutic option to improve prognosis and block tumor progression (Nagini, 2012). Thus, the aim of this work is to analyze the association between RECK gene single-nucleotide polymorphisms (rs16932912) and cirrhosis, HCC susceptibility.

## METHODOLOGY

### *Study subjects and Samples collection*

The study was conducted on 90 HCV patients divided into three groups, group I include thirty HCV patients, group II include thirty HCV patients developing (HCC) and group III include thirty HCV patients developing liver cirrhosis in addition to twenty healthy individuals as a control group. The blood samples were collected from patients at National Hepatology & Tropical Medicine Research Institute.

The HCV cases were diagnosed by polymerase chain reaction (PCR) and using a commercially available Quantikine enzyme linked immunosorbent assay (ELISA) kit provided by (R&D Systems, USA) following the manufacturer's recommendations, while the HCC was diagnosed by Alfa-fetoprotein (AFP) test, in addition to computed tomography and the liver cirrhosis was diagnosed with abdominal sonography.

Liver enzymes, including ALT (alanine aminotransferase), AST (aspartate aminotransferase), serum albumin (Alb), serum bilirubin including total bilirubin (T BIL), direct bilirubin (D BIL) international normalized ratio of prothrombin time (INR), white blood cells (WBC), hemoglobin (Hb), fasting blood sugar and Alpha fetoprotein (AFP) were measured. An informed written consent was obtained from each individual after approval of National Hepatology & Tropical Medicine Research institute ethical committee.

### *Histopathological study*

The liver biopsy specimens were collected intraoperative from cirrhotic and HCC patients. Specimens were fixed in formalin embedded then sectioned and stained by Haematoxylin and

Eosin for routine histological examination to detect the fibrosis score. Histopathological grading and staging were performed according to Modified Kondell's Score.

### *Genomic DNA extraction from whole blood*

DNA was extracted from all patients and control groups using: Genomic whole blood extraction kit (Qiagen, Valencia, CA) according to the instructions of the manufacturer. The DNA was dissolved in TE buffer [10 mM Tris (pH 7.8), 1 mM EDTA] and the DNA purity and concentration were done to all samples using Nano drop. All extracted DNA samples were stored at -20°C and used as templates for Real-time (PCR).

### *Real-time PCR*

The allelic discrimination of the RECK genes rs16932912 polymorphisms was assessed using ABI StepOne™ Real-Time PCR System (Applied Biosystems) and analyzed using SDS v3.0 software (Applied Biosystems), using the TaqMan assay (assay IDs: C\_27084758\_10)

The final volume for each reaction was 20µL, containing 10µL TaqMan Genotyping Master Mix, 0.5µL TaqMan probes mix, and 1µL genomic DNA then completed with distilled water up to 20µL. The real-time PCR reaction included an initial denaturation step at 95°C for 10 min, followed by 40 cycles, each consisting of 95°C for 15 second 60°C for 1 min.

### *Statistical analysis*

Data were analyzed using mini tab 17. Chi-square test (Fischer's exact test) was used to examine the relation between quantitative variables. A P-value less than 0.05 was considered significant.

## RESULTS

Mean value of age between the studied groups were (HCV: 62.4±9.16; HCC-HCV: 59.47±8.79; HCV-LC: 51.73±9.59; control: 48.4±14.5) while gender distribution were in HCV: 20 males (67%) and 10 females (33%); HCC-HCV: 22 males (73%) and 8 females (27%); HCV-LC: 13 males (43%) and 17 females (57%); control: 13 males (65%) and 7 females (35%) with non-significant value (P=0.07, 0.04, 0.03), respectively. RECK rs16932912 mutant genotypes GG/AG showed no significant value in HCC compared to wild type (P=0.373) but this mutant genotype was higher in HCV cirrhotic group than other groups (P=0.001) these results were shown in Table (2) and Figure (1-2).

The distribution frequency of clinical statuses and RECK genotypes frequencies in HCC patients were estimated to clarify the role of RECK gene polymorphisms and viral status of all patients groups, including hepatitis B surface antigen (HBsAg), antibody to HCV (anti-HCV). There was no observed significant association between rs16932912 gene polymorphisms and viral status of all patients groups. Table (4) showed the fibrosis score among the studied groups.

## DISCUSSION

Hepatocellular carcinoma is one of the leading causes of cancer-related deaths in the world and in Egypt. The incidence is expected to rise over the next decades owing to the increasing prevalence of chronic liver diseases, especially those due to underlying viral hepatitis or non-alcoholic steatohepatitis.

**Table 1.** Primer sequences and PCR-RFLP conditions for amplification of RECK SNPs

SNP	Sequences	Product	Enzyme
<b>RECK rs16932912</b>	5'-TGGAGATTGTTGATGGTCTC-3'(forward) 5'-CGGTACACAATGCTCAATAC-3' (reverse)	G/G: 353 bp A/A: 250 bp,103 bp	Tfil

**Table 2.** Rate of RECK (rs16932912) genotypes among HCV, HCC and LC patient groups

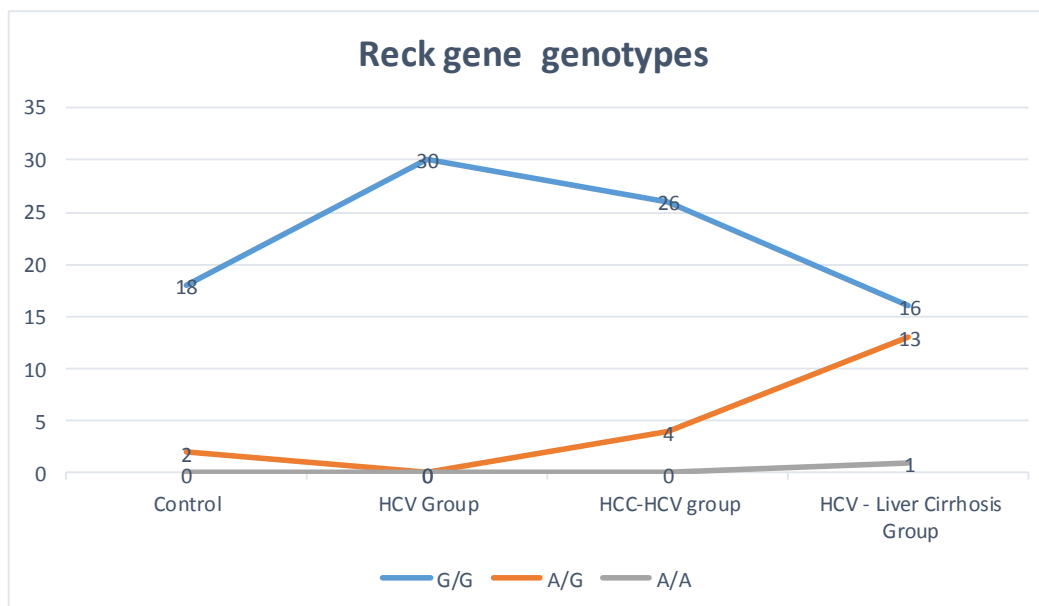
Variable	HCV Group n = 30	HCC - HCV Group n = 30	HCV - Liver Cirrhosis Group n = 30
Wild G/G	30 (100 %)	26 (86.7 %)	16 (53.3%)
Mutant heterozygous A/G	0	4 (13.3 %)	13 (43.3%)
Mutant homozygous A/A	0	0	1 (3.4 %)
All mutant A/G + A/A	0	4 (13.3 %)	14 (46.7 %)
P-Value		0.373	0.001

**Table 3.** RECK gene frequencies in all studied groups regarding viral Status

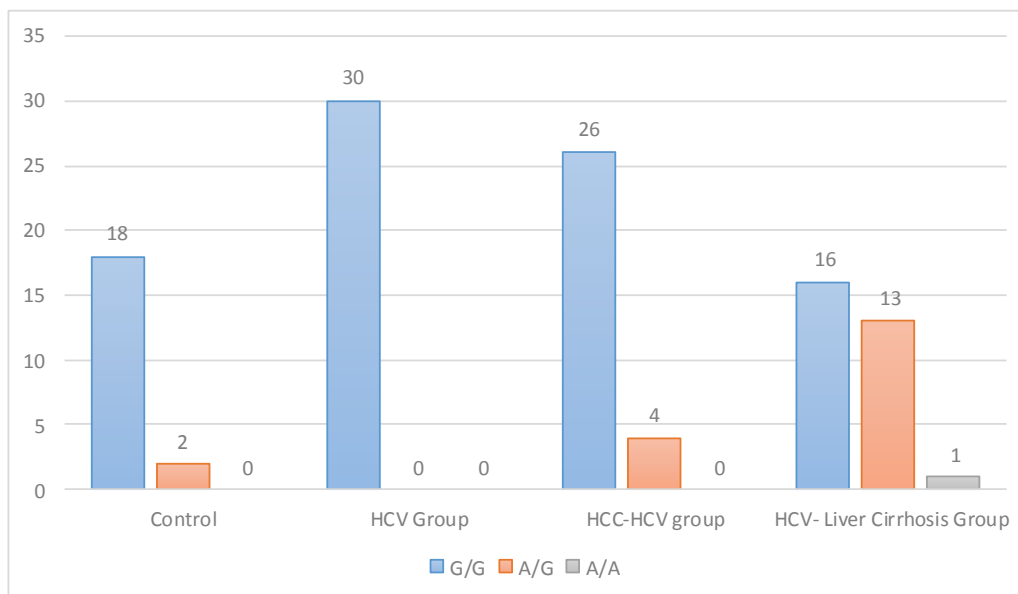
	Control		HCV Group		HCC-HCV Group		HCV - Liver Cirrhosis Group		
Variable	G/G n = 18	A/G n = 2	G/G n = 30	A/G n = 0	G/G n = 26	A/G n = 4	G/G n = 16	A/G n = 13	A/A n = 1
<b>HBsAg</b>	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
Positive	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Negative	18 (100%)	2 (100%)	30 (100%)	0 (0%)	26 (100%)	4 (100%)	16 (100%)	13 (100%)	1 (100%)
<b>HCV Ab</b>	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
Positive	0 (0%)	0 (0%)	30 (100%)	0 (0%)	26 (100%)	4 (100%)	16 (100%)	13 (100%)	1 (100%)
Negative	18 (100%)	2 (100%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)

**Table 4.** Fibrosis score among the studied groups

Variable	HCV Group		HCC - HCV Group		HCV- Liver Cirrhosis Group		
	G/G n = 30	A/G n = 0	G/G n = 26	A/G n = 4	G/G n = 16	A/G n = 13	A/A n = 1
<b>Fibrosis</b>	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
<b>3/6</b>	11 (36.7%)	0 (0%)	6 (23.3%)	1 (25%)	4 (25%)	5 (38.5%)	0 (0%)
<b>4/6</b>	3 (10%)	0 (0%)	3 (10%)	1 (25%)	1 (6.25%)	1 (7.7%)	0 (0%)
<b>5/6</b>	16 (53.3%)	0 (0%)	17 (56.7%)	2 (50%)	11 (68.75%)	7 (53.8%)	1 (100%)



**Figure 1.** Comparison between all studied groups as regards the rate of RECK (rs16932912) genotypes  
**GG:** wild homozygous **AA:** mutant heterozygous



**Figure 2.** Rates of RECK (rs16932912) genotypes among all studied groups.

Currently, a multitude of markers are available for the diagnosis of hepatocellular carcinoma. However, none of these has adequate sensitivity and specificity. For example, alpha fetoprotein, the most widely used serum marker for the diagnosis of HCC in clinical practice has a sensitivity and specificity of 41-65% and 80-94% respectively (Tara and Sitki Copur, 2012).

A high Reversion-inducing-cysteine-rich protein with Kazal motifs (RECK) mRNA expression - which considered as a tumor suppressor gene in HCC tumor tissues from patients

with better survival and less invasive clinicopathologic features (Furumoto et al., 2001).

The genomic structure for the RECK gene has been identified on the chromosome region 9p13→p12. The RECK gene includes 21 exons and 20 introns, and 13 SNPs were identified. Among 13 SNPs, rs16932912 was found within the coding sequence in exons 9 (Eisenberg et al., 2002). These polymorphisms are non-synonymous and changed amino acid sequence as well as RECK protein structure. For example, Valine changes to Lysine in rs16932912 SNP (Chung et al., 2012).

This study evaluated RECK rs16932912 genotypes frequencies on HCC susceptibility and its relation to various clinical and laboratory data. RECK rs16932912 mutant genotypes GG/AG frequencies showed no higher risk of HCC compared to wild type individuals ( $P=0.373$ ) with significance value among liver cirrhosis group ( $P=0.001$ ).

High significant levels of ALT, AST, AFP and ALB ( $P<0.01$ ) among the rs16932912 mutant AG/AA genotypes versus wild GG genotype except albumin had no significant value in HCV-liver cirrhosis group ( $P=0.1856$ ). Frequencies of anti-HCV and liver cirrhosis were no significantly different between the rs16932912 wild genotype GG and mutant genotypes AG/AA. This finding was in parallel conjunction with that concluded by Chung et al. (2012) suggesting that RECK gene rs16932912 polymorphism not a risk factor increasing HCC susceptibility and distant metastasis.

Previous researches confirm the relationships of RECK expression and gene status with tumour metastasis and prognosis where promoter hypermethylation silencing of RECK mRNA was associated with poor survival in HCC (Zhang C et al., 2012). In oral cancer patients, those who had the RECK polymorphism had a higher risk of neck lymph node metastasis than wild type carriers (Chung et al., 2011). Low RECK expression colorectal cancer (Takeuchi et al., 2004), esophageal cancer patients (Li et al., 2007) and non-small cell lung cancer (Chang et al., 2007) exhibited more lymph node metastasis.

Experimental studies showed that RECK can suppress tumor invasion, metastasis, and angiogenesis (Zhang Y et al., 2012). This might find an explanation by Stenzinger et al. (2012) who reported that patient prognosis is determined in most solid cancers by the extent of local invasion and the presence of lymph node and distant metastases. The invasive potential of a tumour depends on the ability to degrade extracellular matrix proteins, for example, by MMPs. RECK being an important inhibitor of MMPs, decreased RECK expression was an independent prognostic factor of poor survival.

Murai et al., (2010) reported that the levels of residual RECK in resected tumours often correlate with better prognosis and that forced expression of RECK in cancer cells suppresses tumour angiogenesis, invasion, and metastasis in xenograft models. RECK is therefore a promising marker for benignancy and a potential effector in cancer therapy. The role of RECK has been demonstrated in several other malignancies. In pancreatic carcinoma, an inverse correlation between RECK expression and MMP-2 activation in the tumor tissues, as well as their invasive potentials, was found. Thus, it is suggested that RECK plays an active role in suppressing malignant phenotypes of pancreatic cancer cells and can be used as a prognostic indicator in these patients (Bloomston et al., 2002).

Our study showed that RECK promoter rs16932912 polymorphism was associated with no risk of HCC susceptibility and lymph node metastasis. RECK promoter rs16932912 polymorphism has no significant influence on the occurrence and progression of HCC cancer.

Additional studies with larger sample sizes are needed to validate the genetic effects of various RECK polymorphisms on HCC. Further studies investigating and correlating different RECK genotypes with RECK expression by immune histochemistry (IHC) analyses of tumour samples are recommended. Further researches are recommended evaluating RECK as a promising prognostic marker, potential therapeutic agent and molecular target for cancer therapy. Previous research conducted by Takahashi et al. (1998)

confirmed the relationships of RECK expression and Gene status with tumor metastasis. In their study, they artificially restored RECK expression in tumor cells, in which RECK was undetectable. This greatly suppressed their invasive and metastatic potentials (Takahashi et al., 1998).

Analysis of the distribution of RECK genotypes among the HCC patients and controls showed a higher frequency of G/A genotypes in HCV-HCC patients and HCV-Liver cirrhosis (13.3% and 46.7%, respectively) compared to the healthy controls (6%) and this difference was statistically significant. Thus, in our study the presence of at least one Polymorphic A allele increases the susceptibility to HCC compared to the G/G wild-type carriers.

Child-Pugh class A to C is used to assess the prognosis of chronic liver disease. The score employs five clinical measures of liver disease that are total bilirubin, serum albumin, prothrombin time, ascites, and hepatic encephalopathy (Pugh et al., 1973). Our study showed that the presence of A/G genotype was associated with prognosis in HCV-HCC and HCV-LC patients.

## CONCLUSION

Our study showed that the presence of A/G genotype was associated with prognosis in HCV-HCC and HCV-LC patients so we recommend by further studies on a larger scale.

## AUTHORS' CONTRIBUTIONS

**Ingy Badawy:** Contributed reagents/materials, carried out the molecular genetic studies and lab tests and manuscript writing.

**Marwa K. Darwish:** Carried out the extraction steps and manuscript writing.

**Omar Samir:** Performed statistical analysis.

**Yassin Baraqouni:** Performed the experiments.

Mohamed Mahmoud Nassef: Performed the experiments.

**Hosam E Elshafey:** participated in study design and coordinated and helped in patients selection.

**Seham M. Mahoud:** participated in study design and helped in patients selection.

**Hala H. El Deeb:** Carried out the molecular genetic studies and manuscript writing.

All authors read and approved the final manuscript.

## COMPETING INTERESTS

Authors have declared that no competing interests exist. The authors alone are responsible for the content and writing of the paper. The authors did not receive any funds from any source.

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