ISSN NO. 2320-5407

Journal homepage: http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

RECK gene polymorphisms in hepatocellular carcinoma: association with susceptibility and clinicopathologic features in Egyptian patients

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Manuscript Info

Manuscript History:

Received: 12 December 2014
Final Accepted: 22 January 2015
Published Online: February 2015

Key words:
RECK, HCC, rs16932912, rs11788747, PCR-RFLP

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Abstract

Background

The RECK (reversion-inducing cysteine rich protein with Kazal motifs) gene was initially isolated as a transformation suppressor gene. RECK down-regulation has been confirmed in numerous human cancers. The aim of this study was to investigate the association of RECK single-nucleotide polymorphisms (SNPs) with hepatocellular carcinoma (HCC) susceptibility and clinicopathologic characteristics in Egyptian patients.

Methodology

A total of 50 HCC cancer patients and 30 cancer-free controls were analyzed for RECK rs16932912 and rs11788747 genotyping using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay.

Results

RECK rs11788747 mutant genotypes AG/GG showed a 2.779 fold (95% CI: 1.091–7.080) higher risk of HCC compared to wild genotype (p=0.030) with a higher risk of lymph node metastasis (p = 0.034) and significantly higher levels of ALT, AST, and ALP (p=0.011, 0.001 and 0.003), respectively. RECK rs16932912 gene showed no statistical significance.

Conclusions

This study revealed the role of RECK rs11788747 polymorphism on HCC susceptibility and prognosis in Egyptian patients.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer globally [(1)]. HCC is the third most common cause of cancer mortality [(2)]. The development of HCC is a multistep and complex process. Multiple enviromental 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 [(3), (4)].

The RECK (reversion-inducing cysteine rich protein with Kazal motifs) protein was initially discovered by its ability to induce reversion in ras-activated fibroblasts. The key action of RECK is to inhibit matrix metalloproteinases (MMPs) involved in breakdown of the extracellular matrix (ECM), and angiogenesis. To this effect, it plays important physiological roles in embryogenesis and vasculogenesis [(5)]. RECK modulates the function of MMPs by directly inhibiting their proteolytic activity and modulating the endocytic pathway. Hence, RECK could play a key role in tumour suppression by involving in the regulation of angiogenesis and maintenance of vascular integrity [(6)]. RECK is essential for normal development and is a key mediator of tissue remodelling and stabilization of tissue architecture. Down regulation of RECK documented in a wide range of malignant neoplasms correlates with poor prognosis, and tumour metastasis [(7)]. There are almost certainly pathways by which RECK is down regulated in cancer. Hypoxia induces RECK down regulation through the recruitment of histone deacetylase 1 (HDAC1) and hypoxia inducible factor-1alpha (HIF-1α) to the second reverse hypoxia response element (rHRE2) site in the RECK promoter; and the inhibition of hypoxic RECK silencing would be a therapeutic and preventive target for early tumorigenesis. The promoter hypermethylation is associated with silencing of tumour suppressor genes, which is the most recognized epigenetic disruption in human tumours [(8)]. It is implied that the promoter methylation of RECK could play a particularly important regulatory role for RECK expression in carcinogenesis. The molecular mechanisms behind RECK transcriptional regulation are clearly complex, and are still yet to be fully defined [(9)]. RECK down regulation, promoter hypermethylation and decreased RECK expression have been confirmed in many human cancers including pancreatic cancer [(10), (11)], breast cancer [(12), (13)], lung cancer [(14), (15)], colorectal cancer [(16), (17)], gastric cancer [(18)], prostate cancer [(19)], oral cancer [(20)-(22)], oesophageal cancer [(23)] and osteosarcoma [(24)]. The RECK gene is a common negative target for oncogenic signals that act on the specificity protein 1 (Sp1) binding site of the RECK promoter. Both natural and synthetic agents have been identified as up regulators of RECK. Several strategies have been proposed to enhance RECK expression including forced expression of RECK, use of mimetics, recombinant peptides, microRNA antagonists, and gene therapy. Up regulation of RECK could be a valuable therapeutic option to improve prognosis and block tumour progression [(7)].

Our study performs a case-control study for two single-nucleotide polymorphisms (SNPs) rs16932912 and rs11788747 located on the RECK gene to analyze their association with HCC susceptibility and clinicopathologic characteristics in Egyptian population.

Subjects and methods

Study subjects

The study was performed at the period from June 2012 to December 2013. The included patients were gathered from National Cancer Institute of Egypt. The cases and controls (50 HCC and 30 apparently healthy control individuals) appeared to be well matched on age and sex.

Inclusion criteria

The diagnosis of HCC was based on medical history, physical examination and raised level of alpha fetoprotein (AFP). Diagnosis was confirmed by triphasic CT and CT guided biopsy histopathological examination.

Exclusion criteria

Patients with prior history of other cancers. Controls with any personal or family history of malignancies.

HCC patients were staged according to the TNM staging system of the American Joint Committee on Cancer (AJCC) (2002) [(25)]. Liver cirrhosis was diagnosed with liver biopsy, abdominal sonography and biochemical evidence of liver parenchymal damage with endoscopic esophageal or gastric varices. The patients' clinicopathological characteristics and laboratory data, such as clinical staging, Child-Pugh grade, lymph node metastasis, distant metastasis, antibody to HCV (anti-HCV), liver cirrhosis, alpha fetoprotein (AFP), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatise (ALP) were verified.

Before the conduction of this study, approval from the Scientific Research Committee, National Cancer Institute of Egypt was obtained, and informed written consent was obtained from each individual. Data confidentiality was preserved according to the Revised Helsinki Declaration of Bioethics (2008) [(26)].

Genomic DNA extraction

Blood samples were anticoagulated with ethylenediamine tetra acetic acid (EDTA).The genomic DNA was extracted from each blood sample using Gene JET Genomic DNA purification kit (Cat No.# K0721, Lot/ 167300 , Fermentas Life Sciences) according to the manufacturer's instructions.

Polymerase chain reaction-restriction fragment length polymorphism

The RECK rs16932912 and rs11788747 gene polymorphisms were determined by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay. The PCR primers for analysis of the RECK gene polymorphisms [(27)] are described in table 1. Amplification was carried out on ARKTIK Thermal Cycler. PCR assay for each gene was performed for each sample in a final reaction volume of 25 µL, using 5 µL DNA, 12.5 µL Maxima Hot Start Green PCR Master Mix (Cat No.# K1061, Lot/ 242047), 1 µL forward primer, 1 µL reversed primer, together with 5.5 µL distilled water (DW).The PCR cycling conditions were 5 min at 94°C followed by 35 cycles of denaturation 1 min at 94°C, annealing 1 min at 60°C, and extention 2 min at 72°C, with a final extention step at 72°C for 15 min[(27)]. The PCR products were subjected to digestion at 37°C overnight with the restriction enzymes *Tfi*I (Cat No. #FD1784, Lot:00121579, Thermo Fisher Scientific Inc.) for RECK rs16932912 and *Rsa*I (Cat No.#FD1124, Lot:00118446, Thermo Fisher Scientific Inc.) for RECK rs11788747[(27)]. The products were then resolved on 2% agarose gel electrophoresis containing ethidium bromide, then visualized using UV transilluminator. DNA molecular weight marker 50-500 bp Ladder (Cat No. #SM1213, Lot. 00132289, Thermo Fisher Scientific Inc.) was used to assess the size of PCR-RFLP products. The PCR products after digestion are demonstrated in table 1. The PCR products for RECK rs16932912 and RECK rs11788747 are presented in Figure 1 and Figure 2, respectively.

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

SNP	Sequences	Product	Enzyme
RECK rs16932912	*5′-TGGAGATTGTTGATGGTCTC-3′ (forward)* *5′-CGGTACACAATGCTCAATAC-3′ (reverse)*	GG: 353 bp AA: 250 bp, 103 bp	*Tfi*I
RECK rs11788747	*5′-GTAGAAGAAGTGACTCATCC-3′ (forward)* *5′-ATCTCACTCCGAAGATAACC-3′ (reverse)*	AA: 242 bp GG: 140 bp, 102 bp	*Rsa*I

Statistical analysis

Data were analyzed using IBM SPSS advanced statistics version 20 (SPSS Inc., Chicago, IL). Chi-square test (Fisher's exact test) was used to examine the relation between qualitative variables. For quantitative data, comparison between two groups was done using either student t-test or Mann-Whitney test (non-parametric t-test) as appropriate. Odds ratio (OR) with it 95% confidence interval (CI) were used for risk estimation. Spearman-rho method was used to test correlation between numerical variables. A p-value < 0.05 was considered significant [(28)].

Results

In our study the included patients were gathered from National Cancer Institute of Egypt. The cases and controls (50 HCC and 30 healthy control individuals) appeared to be well matched on age and sex where the distributional differences of age (HCC: 58.2±5.4; control: 57.3±5.8) and sex (HCC: 38 males (76.0%) and 12 females (24.0%); control: 20 males (66.7%) and 10 females (33.3%)) were insignificant (P = 0.466 and 0.367), respectively.

Table 2 shows the association between RECK rs16932912 and rs11788747 genotypes frequencies with HCC. RECK rs11788747 mutant homozygous genotype GG and all mutant genotypes AG/GG frequencies showed 9.562 fold (95% CI: 1.907-47.960, p=0.006) and 2.779 fold (95% CI: 1.091–7.080, p=0.030), respectively higher risk of HCC compared to wild genotype. RECK rs16932912 gene showed no statistical significance.

Table 2: Odds ratio (OR) and 95% confidence interval (CI) of HCC associated with RECK genotypic frequencies

Gene	Lung cancer n (%)	Controls n (%)	p-value	Odds ratio	95%confidence interval
rs16932912 Wild type *GG*	36(72%)	18(60%)	-	1	1

Mutant heterozygous *GA*	4(8%)	10(33.3%)	0.144	0.200	(0.055-1.727)
Mutant homozygous *AA*	10(20%)	2(6.7%)	0.268	2.500	(0.495-12.635)
All mutant *GA/AA*	14(28%)	12(40%)	0.267	0.583	(0.224-1.518)
rs11788747 Wild type *AA*	16(32%)	17(56.7%)	-	1	1
Mutant heterozygous *AG*	16(32%)	11(36.7%)	0.406	1.545	(0.553-4.317)
Mutant homozygous *GG*	18(36%)	2(6.7%)	0.006	9.562	(1.907-47.960)
All mutant *AG/GG*	34(68%)	13(43.3%)	0.030	2.779	(1.091-7.080)

To clarify the role of RECK gene polymorphisms in progression of HCC, RECK genotypes frequencies were estimated in the clinicopathologic states of HCC patients, including TNM clinical staging, Child-Pugh grade, lymph node involvement, distant metastasis, antibody to HCV (anti-HCV), and liver cirrhosis. Significantly different frequencies between RECK rs116932912 wild genotype GG and mutant genotypes AG/AA were observed as regards anti-HCV ($p = 0.013$) and liver cirrhosis ($p = 0.027$). RECK rs11788747 mutant genotypes AG/GG frequencies showed a 0.222 fold (95% CI: 0.051–0.952) higher risk of lymph node metastasis compared to wild genotype ($p = 0.034$) (table 3).

Table 3: Clinicopathological states and RECK genotypic frequencies in HCC patients

Clinicopathological states	All mutant N (%)	Wild type N (%)	*p*-value	Odds ratio	95%confidence interval
rs16932912					
TNM clinical staging					
III,IV	8(40%)	12(60%)			
I,II	6(20%)	24(80%)	0.123	2.667	0.753-9.450
Child-Pugh grade					
B or C	6(37.5%)	10(62.5%)			
A	8(23.5%)	26(76.5%)	0.305	1.950	0.539-7.052
LN metastasis					
Present	12(30%)	28(70%)	0.529	0.583	0.108-3.163
Absent	2(20%)	8(80%)			
Distant metastasis					
Present	3(33.3%)	6(66.7%)	0.694	1.364	0.290-6.415
Absent	11(26.8%)	30(73.2%)			

Anti-HCV					
Present	14(36.8%)	24(63.2%)			
Absent	0(0%)	12(100%)	0.013	0.632	0.495-0.805
liver cirrhosis					
Present	14(35%)	26(65%)			
Absent	0(0%)	10(100%)	0.027	0.650	0.518-0.816
rs11788747					
TNM clinical staging					
III,IV	12(60%)	8(40%)			
I,II	22(73.3%)	8(26.7%)	0.322	0.545	0.163-1.822
Child-Pugh grade					
B or C	10(62.5%)	6(37.5%)			
A	24(70.6%)	10(29.4%)	0.567	0.694	0.198-2.430
LN metastasis					
Present	30(75%)	10(25%)			
Absent	4(40%)	6(60%)	0.034	0.222	0.052-0.951
Distant metastasis					
Present	7(77.8%)	2(22.2%)			
Absent	27(65.9%)	14(34.1%)	0.487	1.815	0.332-9.923
Anti-HCV					
Present	26(68.4%)	12(31.6%)			
Absent	8(66.7%)	4(33.3%)	0.910	1.083	0.272-4.312
liver cirrhosis					
Present	26(65%)	14(35%)			
Absent	8(80%)	2(20%)	0.363	0.464	0.087-2.492

This study also analyzed the levels of laboratory data ALT, AST, and ALP in association with RECK genotypes frequencies. Table 4 shows that the levels of ALT, AST, and ALP were significantly higher among the rs11788747 mutant AG/GG genotypes versus wild AA genotype (p=0.011, 0.001 and 0.003), respectively.

Table 4: Association of RECK genotypic frequencies with HCC laboratory data

Characteristic	ALT (IU/L)	AST (IU/L)	ALP(IU/L)
rs16932912			
Wild GG	77.7±38.1	134.4±67.7	209.1±145.5
All mutant GA/AA	85.3±36.5	139.1±81.9	156.5±19.5
p-value	0.482	0.854	0.151
rs11788747			
Wild AA	63.1±33.5	92.0±51.4	131.9±21.1
All mutant AG/GG	87.7±37.0	156.3±70.3	223.7±148.4
p-value	0.011	0.001	0.003

Discussion

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 and rs11788747 were found within the coding sequence in exons 9 and 13, respectively [(29)].

This study evaluated RECK rs116932912 and rs11788747 genotypes frequencies on HCC susceptibility and its relation to various clinical and laboratory data. RECK rs11788747 mutant genotypes AG /GG frequencies showed a 2.779 fold higher risk of HCC compared to wild genotype (p=0.030) with a higher risk of lymph node metastasis (p = 0.034) and significantly higher levels of ALT, AST, and ALP (p=0.011, 0.001 and 0.003), respectively. Frequencies of anti-HCV and liver cirrhosis were significantly different between the rs116932912 wild genotype GG and mutant genotypes AG/AA (p = 0.013 and 0.027), respectively.

Chung et al. 2012 [(27)] showed that HCC patients carrying rs11788747 polymorphisms had a higher risk of distant metastasis than wild type carriers ($p=0$ .003) and significantly different ALT levels between its different genotypes (p=0.040) suggesting that RECK gene polymorphism might be 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 [(9)]. In oral cancer patients, those who had the RECK polymorphism had a higher risk of neck lymph node metastasis than wild type carriers [(21)]. Low RECK expression colorectal cancer[(16)], esophageal cancer patients[(30)] and non-small cell lung cancer[(31)] exhibited more lymph node metastasis .

Experimental studies showed that RECK can suppress tumor invasion, metastasis, and angiogenesis [(13)]. This might find an explanation by Stenzinger et al. 2012 [(17)] 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 [(32)] 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.

In conclusion, our study showed that RECK promoter rs11788747 polymorphism was associated with higher risk of HCC susceptibility and lymph node metastasis. RECK promoter rs11788747 polymorphism could have a 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 immunohistochemistry (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.

Conflicts of interest: The authors have no conflicts of interest to declare. The authors alone are responsible for the content and writing of the paper. The authors did not receive any funds from any source.

Ethical considerations: All patients and healthy controls included in this study gave their informed consent and approval upon participating in the study. Neither patients' names nor photos were included in this study.

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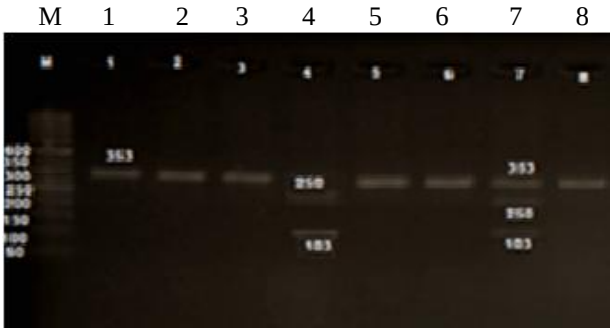

Figure 1: PCR-RFLP analysis of RECK rs16932912 gene polymorphism using *Tfi*I restriction enzyme analysis by agarose gel electrophoresis
Lanes 1, 2, 3, 5, 6, 8: DNA from GG homozygous wild type represented by one band at 353 bp.
Lanes 7: DNA from GA heterozygous mutant type represented by three bands at 353, 250 and 103 bp.
Lane 4: DNA from AA homozygous mutant type showing two bands at 250 and 103 bp.
M: DNA molecular weight marker (50–500 bp)

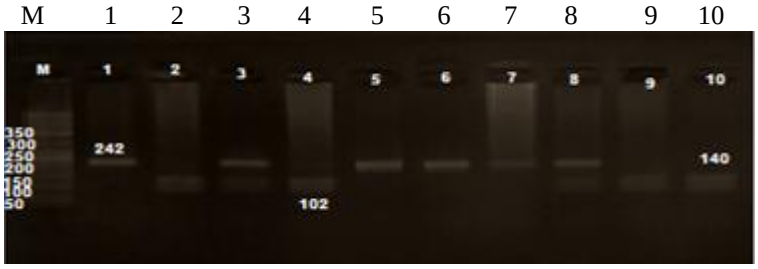

Figure 1: PCR-RFLP analysis of RECK rs11788747 gene polymorphism using *Rsa*I restriction enzyme analysis by agarose gel electrophoresis
Lanes 1, 5, 6, 7: DNA from AA homozygous wild type represented by one band at 242 bp.
Lanes 3, 8: DNA from AG heterozygous mutant type represented by three bands at 242, 140 and 102 bp.
Lane 2, 4, 9, 10: DNA from GG homozygous mutant type showing two bands at 140 and 102 bp.
M: DNA molecular weight marker (50–500 bp)