



## Role of rs 1800625 and 63bp deletion RAGE Gene Polymorphisms in Hepatocellular Carcinoma Progression

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### ABSTRACT

Hepatocellular carcinoma (HCC) is a common liver malignancy, whose heterogeneous occurrence indicates genetic dissimilarities between people in the main risk factors. Advanced glycation end product receptor (RAGE) is a multiligand receptor implicated in several pathogenic conditions, including cancer. In this study, the impact of RAGE gene polymorphisms on the susceptibility to hepatocarcinogenesis was explored. To estimate the effect of gene variations of RAGE on the progress of HCC caused by any viral infection and to examine the relationship between a RAGE and the risk and progression of HCC. Single-nucleotide polymorphism (SNP; rs1800625) (-429T>C), and the 63 bp deletion (-407 to -345) in the 5' flanking region of the RAGE gene were investigated among 90 HCV patients divided into 3 groups and 20 healthy controls. The HCV cases were diagnosed by polymerase chain reaction (PCR) while the HCC was diagnosed by Alfa-fetoprotein (AFP) test in addition to computed tomography. Liver cirrhosis was diagnosed with abdominal sonography. The study detected a significant association of rs1800625 with the increased risk of HCC also majority of HCC patient (86.7%) showed 63 bp deletion polymorphism (407 – 345), our data suggest a correlation of RAGE gene polymorphism rs1800625 with the early stage of liver tumorigenesis and implicate its protective role in the progression of HCC.

### 1. Introduction

Hepatocellular carcinoma (HCC, also called malignant hepatoma) is the most common type of liver cancer [1]. It is the fifth most common neoplasm, the major cause of death in patients with cirrhosis, and the third most common cause of cancer-related death in the world [2]. HCC carcinogenesis is a complex process linked with various risk factors, counting exposure to aflatoxin B, hepatitis B virus or hepatitis C virus (HCV) (HBV) chronic infection, excessive alcohol and tobacco consumption, and diabetes [3].

Egypt has the highest prevalence of HCV in the world (predominantly genotype 4), which has been attributed

to previous public health eradication schemes for schistosomiasis [4]. Even higher HCV infection rates, up to 60%, have been reported in older individuals, in rural areas such as the Nile delta, and in lower social classes [5]. In Egypt, HCC was reported to account for about 4.7% of CLD patients [6]. Between 1993 and 2002, there was an almost two-fold increase in HCC amongst chronic liver patients in Egypt [7].

Several evidences have suggested that single-nucleotide polymorphisms (SNPs) may be linked to liver carcinogenesis in defined populations [8,9]. These studies spotlighted disparities within individual genomes that control oxidative stress, DNA repair, cell

signaling, immune and inflammatory responses as genetic tendencies toward hepatic-tumorigenesis and somewhat clarified the detected differences in the risk of HCC incidence [10].

Advanced glycation end products, known as AGEs, are substances that can be a factor in the development or worsening of many degenerative diseases, such as diabetes, atherosclerosis, chronic renal failure, and Alzheimer's disease. These harmful compounds can affect nearly every type of cell and molecule in the body and are thought to be one factor in aging and in some age-related chronic diseases.

Receptor for advanced glycation end products (RAGE) is a multiligand cell surface receptor that belongs to the immunoglobulin superfamily [11]. RAGE expression is usually low in the majority of healthy adult tissues, but its levels are elevated under pathological conditions such as diabetes, cardiovascular disease, Alzheimer's disease, and cancer [12, 13].

RAGE activation by various ligands have been reported to increase oxidative stress generation and subsequently evoke inflammatory, proliferative, angiogenic, fibrotic, thrombogenic, and apoptotic reactions in numerous cell types via activation of diverse intracellular signaling pathways such as nuclear factor- $\kappa$ B (NF- $\kappa$ B), mitogen-activated protein kinase (MAPK), Janus kinase-signal transducers and activators of transcription (JAK-STAT), and phosphoinositol 3 kinase [14, 15]. RAGE is expressed in hepatic cells, hepatocytes and hepatoma cells [16]. There is accumulating evidence that activation of RAGE signaling pathways in the liver could contribute to the development and progression of numerous types of hepatic disorders [17].

Moreover, rs1800625 SNP of the RAGE gene has been shown to be widely linked with the progress of many malignancies, as lung, kidney, and oral cancers [18 - 20]. Also, 63 bp deletion; a rare variant; is known to increase the transcriptional activity of RAGE [21].

We aimed in this study to evaluate the impact of gene variations of RAGE on the development of HCC caused by viral infection, and to observe a possible association of a RAGE SNPs with the risk and progression of HCC.

## 2. Material and methods

### 2.1 Subjects

The study was performed in the Research unit at Oncology Diagnostic Unit, Faculty of Medicine, Ain Shams University. The study was conducted on 90 HCV patients divided into three groups:

Group I included 30 HCV patients who developed Hepatocellular carcinoma (HCC). Group II included 30 HCV patients with liver cirrhosis. Group III: included 30 chronic HCV patients without cirrhosis, in addition 20 healthy individuals were enrolled as a control group (Group IV). Patients suffering from any other disease were omitted from our study as infection with Schistosomiasis or other endemic disease. An informed written consent was obtained from each individual and this study was approved by Ain Shams University, Faculty of Medicine Ethical Committee.

### 2.2 Samples

The blood samples were collected from patients attending Oncology Diagnostic Unit. The HCV cases were diagnosed by polymerase chain reaction (PCR) while the HCC ones were diagnosed by Alfa-fetoprotein (AFP) test in addition to computed tomography. Liver cirrhosis was diagnosed with abdominal sonography.

DNA was extracted from all patients and control groups using genomic peripheral mononuclear cells (PMNCs) blood extraction kit (Qiagen, Valencia, CA) according to the instructions of the manufacturer. All extracted DNA samples were stored at -20°C and used as templates for Real-time (PCR).

### 2.3 RAGE Genotyping

The allelic discrimination of the RAGE SNPs was detected in all participants using the TaqMan assay for (rs1800625) (-429T > C) and the 63 bp deletion (-407 to -345) in the 5' flanking region. Both were assessed using ABI StepOne TM Real-Time PCR System (Applied Biosystems) and analyzed using SDS v3.0 software (Applied Biosystems). rs1800625 was amplified using forward primer: 5'-CCTGGGTTTAGTTGAGAATTTTTT-3', reverse primer: 5'-ATTCATGCCTTGGGA CAAGAG-3'. Real-time PCR reaction program for RAGE SNPs (rs1800625) was initial denaturation at 95°C for 10 minutes, followed by 40 cycles amplification (denaturation at 95°C for 15 seconds, annealing at 60°C for 30 seconds, elongation at 72°C for 30 seconds and finally 60°C for 1 minute).

In addition, the 63 bp deletion (-407 to -345) allelic polymorphisms detection [22] was initiated at 94 °C for 1.5 minutes, then 35 cycles (denaturation at 95 °C for 15 seconds, annealing at 60 °C for 30 seconds, elongation for 30 seconds at 72 °C, and finally extension for 10 minutes at 72 °C). The products were separated on a 3 % agarose gel and then stained with ethidium bromide. 327bp and 390bp are the expected product sizes for the deleted and major allele, respectively.

#### 2.4 Statistical analysis

Data were subjected to statistical analysis and tests of significance were performed using the statistical package SPSS (Statistical Program for Social Science) version 15.0 by applying Nonparametric tests (chi squared test and Fisher's exact probability test). The odds ratios (OR) and 95% confidence intervals (CI) were also calculated. All P values less than 0.05 by the two-tailed test were considered significant. Variables that achieved statistical significance ( $P < 0.05$ ) on univariate analysis were entered into multiple logistic regression analysis to identify significant independent predictive factors. Each variable was transformed into categorical data consisting of two simple ordinal numbers for univariate and multivariate analyses.

### 3. Results

#### 3.1 Demographic and clinical characteristics of the studied cases

Among the present study, non-significant difference was observed in age among all studied group ( $P > 0.05$ ). In the current study, a significant difference ( $P$ -value: 0.001) was observed between the different studied groups considering patient's gender. Meanwhile, females predominate in group II, III, and control, they constitute approximately 80% of all subjects in the same group; males predominate in group I, they constitute 19 (63.3 %) in contrast to 11 (36.7 %) for females Table 1.

Regarding, the subject's residence, a high significant difference was detected between the four participated groups Table 1. The majority of patients who develop HCC (group I) were of resident in rural areas 76.7 %, in contrast to 23.3 % who were of urban residence. In contrary, 60 % and 66.7 % of group II & III patients respectively belong to urban origin. Likewise, urban residence predominates in the healthy control group, it constitutes 70 %. The smoking habits were reported and compared in different studied groups; no significant difference was observed between the four groups regarding smoking habits ( $P > 0.05$ ) Table 1.

**Table 1** Distribution of demographical characteristics in the studied groups

Variable	Subgroups	Studied subjects				P - value	Sig.
		Group I N = 30	Group II N = 30	Group III N = 30	Control N = 20		
Age (years)	Mean ± SD	60.1 ± 6.8	54.9 ± 9.0	52.2 ± 9.0	42.9 ± 11.0	0.072	NS
	Range	50.0 – 75.0	39.0 – 68.0	39.0 – 68.0	17.0 – 57.0		
Gender N (%)	Male	19 (63.3 %)	6 (20 %)	5 (16.7 %)	4 (20 %)	0.001	HS
	Female	11 (36.7 %)	24 (80 %)	25 (83.3 %)	16 (80 %)		
Residence N (%)	Urban	7 (23.3 %)	18 (60 %)	20 (66.7 %)	14 (70 %)	0.001	HS
	Rural	23 (76.7 %)	12 (40 %)	10 (33.3 %)	6 (30 %)		
Smoking N (%)	Smoker	6 (20 %)	4 (13.3 %)	3 (10 %)	2 (10 %)	0.660	NS
	Non-smoker	24 (80 %)	26 (86.7 %)	27 (90 %)	18 (90 %)		

Group I: HCC/HCV positive; Group II: HCV positive with cirrhosis; Group III: HCV positive without cirrhosis

### 3.2 Frequencies of RAGE (rs1800625) gene polymorphism among different studied groups

A cross-tabulation test was conducted to calculate the frequencies of distribution of RAGE gene polymorphism among the four studied groups. The majority of patients who developed HCC "group I" showed variant genotype (83.3 %), Also, the variant RAGE gene constitutes (80 %), (73 %) and (70 %) in groups II, III, and control; respectively. Non-significant difference was detected between the four groups ( $P > 0.05$ ). The distribution of RAGE gene genotype among the different studied groups is presented in Table 2.

### 3.3 Frequencies of 63bp deletion (-407 to -345) in the 5' flanking region RAGE gene polymorphism among different studied groups

A cross-tabulation test was conducted to calculate the frequencies of the 63bp deletion (-407 - 345) in the 5' flanking region of RAGE gene among the four studied groups. The results revealed that the 63 bp deletion was detected in 63 cases (55 patients out of 90 and 8 in control group out of 20) and that the majority of patients who developed HCC "group I" showed variable genotype (87.7 %). In addition, the variant RAGE gene constitutes (67.7 %), (30 %) and (40 %) in-group II, III, control respectively. A high significant difference was detected between the four groups ( $P < 0.001$ ). The distribution of 63bp deletion of RAGE gene among the different studied groups is presented in Table 3.

**Table 2** Genotype Frequencies of RAGE (rs; 1800625) Gene Polymorphisms the studied subjects

Variable	Group I N = 30	Group II N = 30	Group III N = 30	Control N = 20	c <sup>2*</sup>	P - value	Sig
Wild, N (%)	5 (16.7 %)	6 (20 %)	8 (26.7 %)	6 (30 %)	1.622	0.654	NS
Variant, N (%)	25 (83.3 %)	24 (80 %)	22 (73.3 %)	14 (70 %)			

\*Chi-square test NS: non-significant

Group I: HCC/HCV positive; Group II: HCV positive with cirrhosis; Group III: HCV positive without cirrhosis

**Table 3** Genotype Frequencies 63bp deletion of RAGE Gene among different studied subjects

Variable	Group I N = 30	Group II N = 30	Group III N = 30	Control N = 20	c <sup>2*</sup>	P - value	Sig
Wild, N (%)	4 (13.3 %)	10 (33.3 %)	21 (70 %)	12 (60 %)	23.2	0.000	HS
DEL, N (%)	26 (87.7 %)	20 (67.7 %)	9 (30 %)	8 (40 %)			

\*Chi-square test HS: high significant difference ( $p < 0.01$ )

Group I: HCC/HCV positive; Group II: HCV positive with cirrhosis; Group III: HCV positive without cirrhosis

### 3.4 Genotype Distribution of RAGE gene (T/C; rs1800625) polymorphism among different studied groups

In order to determine the predominant genotype of RAGE gene (T/C) polymorphism, we calculated the frequencies of different genotypes among the four investigated groups, the results revealed that the heterozygous TC genotype was the most frequent (56%) in the chronic HCV patients who develop HCC

(group I), followed by the homozygous TT genotype which constitutes 32 %. Likewise, 58 % & 73 % of group II & III respectively showed predominant heterozygous TC genotype. For the healthy control, 50 % of subjects showed homozygous TT genotype, followed by 43 % of heterozygous TC genotype, however, the homozygous CC genotype was observed in one subject only. The distribution of T/C gene polymorphism of RAGE gene among the four studied groups is presented in Table 4.

**Table 4** Genotype Distribution of RAGE rs 1800625 Gene Polymorphisms in the studied subjects

Variable	Total N (%)	Group I N = 30	Group II N = 30	Group III N = 30	Control N = 20	c <sup>2</sup>	P - value	Sig
TT	26 (30.5 %)	8 (32 %)	5 (21 %)	6 (27 %)	7 (50 %)	8.8	0.183	NS
TC	50 (59 %)	14 (56 %)	14 (58 %)	16 (73 %)	6 (43 %)			
CC	9 (10.5 %)	3 (12 %)	5 (21 %)	0 (0 %)	1 (7 %)			
Total variant	85	25	24	22	14			

\*Chi-square test NS: Non-significant difference ( $P > 0.05$ )

Group I: HCC/HCV positive; Group II: HCV positive with cirrhosis; Group III: HCV positive without cirrhosis

### 3.5 Genotype Distributions of RAGE 63bp deletion among different studied groups

A cross-tabulation test was conducted; the 63 bp deletion was detected in 46 subjects (73 %) among 63. Our data also reveal that 85 % of RAGE gene polymorphism observed in group I, are of deletion genotype (22 out of 26), likewise, 75 % (15 out of 20) and 67 % (6 out of 9) deletions were observed in group II and group III, respectively. In contrast, it was found that the 63bp deletion was less frequently distributed among healthy control group (3/8; 38 %). Our results showed that, there was no significant difference between

the different studied groups as regarding the type of 63bp RAGE gene deletion polymorphism ( $P > 0.05$ ) Table 5.

### 3.6 Association between RAGE (T/C rs1800625) gene polymorphism and liver status

A normality test was performed for the serum levels of AFP, AST and ALT in all groups. Higher median levels of AFP and AST were observed in patient who had TT genotype in contrast to non-TT genotype, ( $P > 0.05$ ). On the other hand, the non-TT genotype showed higher serum level of ALT in contrast to the TT genotype. These data are presented in Table 6.

**Table 5** Genotype Distributions of RAGE 63bp deletion in the studied subjects

Variable	Total	Group I	Group II	Group III	Control	c <sup>2</sup>	P - value	Sig
63 bp del	46 (73 %)	22 (85 %)	15 (75 %)	6 (67 %)	3 (38 %)	7.1	0.06	NS

X<sup>2</sup>: \*Chi-square test NS: Non-significant difference ( $P > 0.05$ )

Group I: HCC/HCV positive; Group II: HCV positive with cirrhosis; Group III: HCV positive without cirrhosis

**Table 6** Association of RAGE Genotypic Frequencies with HCC Laboratory Status

Variable	TT (Wild type) N = 26	TC/CC (Variant type) N = 59	c <sup>2</sup>	P - value	Sig
AFP (ng/ml)	115 (8.2 – 4900)	28 (3.3 – 5000)	1.5	0.2	NS
AST (IU/ml)	79 (36 – 141)	40 (15 – 92)	3.4	0.06	NS
ALT (IU/ml)	33 (13 – 76)	47 (13 – 114)	2.2	0.14	NS

\*Chi-square test NS: Non-significant difference ( $P > 0.05$ )

### 3.7 Associations between RAGE (T/C rs1800625) Genotypes and Clinicopathologic characteristics of HCC

In order to determine the impact of C/T RAGE gene polymorphisms detected in group I on the severity of HCC in chronic HCV infected patients, we compared the frequencies of TT and non-TT (TC/CC) genotypes in risk

groups of HCC/HCV positive using log regression analysis. Our results revealed that, there was no significant difference between both genotypes regarding gender (OR: 0.9), Lymph nodes metastases (OR: 2.2), and Barcelona clinic liver cancer (BCLC) score (OR: 1.1). These data are presented in Table 7.

**Table 7** Associations between Polymorphic Genotypes of rs1800625 and Clinico-pathologic characteristics of HCC/HCV positive group

Parameter	Subgroups	RAGE rs1800625		Statistics		
		TT N = 8	TC+CC N = 17	Odd ratio	P value	Sig
Gender	Male	5 (31 %)	11 (69 %)	0.9 (0.2 – 6.0)	0.91	NS
	Females	3 (33 %)	6 (67 %)			
LN metastases	No	7 (31 %)	16 (69 %)	2.2 (0.1 – 42)	0.56	NS
	Yes	1 (50 %)	1 (50 %)			
BLCL score	A	4 (33 %)	8 (67 %)	1.1(0.2 – 6.0)	0.17	NS
	B+C	4 (31 %)	9 (69 %)			

LN: lymph nodes, BLCL: Barcelona clinic liver cancer (BCLC) staging, A: Early stage, B: Intermediate Stage, C: Advanced stage, HCV: Hepatitis C Virus

#### 4. Discussion

According to the Egyptian Demographic Health Survey (EDHS) registry, Egypt is the country which recorded the highest HCV prevalence in the world at 2008 [23]. HCC represent the 2<sup>nd</sup> common malignancy, this was contributed to the underlying chronic liver disease especially the viral infection, which include Hepatitis C virus (HCV) and Hepatitis B Virus (HBV) [24].

Genetic mutations reflect the heterogeneous variations among individuals in the main risk factors, this means that in spite of the carcinogenic predisposition of chronic HCV, the development of HCC in those patients is highly influenced by their genetic predisposition, which further increase the risk in those patients [25].

The receptor for advanced glycosylation end-products (RAGE) is a multi-ligand receptor which has been implicated in the development or worsening of many degenerative diseases, such as atherosclerosis, chronic renal failure, auto-immune diseases, diabetes, cancer, inflammatory disorders and Alzheimer disease. These harmful compounds can affect nearly every type of cell and molecule in the body and are thought to be one factor in aging and in some age-related chronic diseases [26].

In this study, the impact of RAGE gene polymorphisms on the susceptibility to develop HCC in Egyptian patients with chronic HCV infection was investigated, in addition, the association between the RAGE gene mutation and prognosis of HCC has been evaluated. All participants were examined for the RAGE (C/T) rs: 1800625 genotype, moreover, the 60bp

deletion allele (-407 to -345) polymorphism was determined in all subjects in the 5' flanking region of RAGE gene, and the impact of RAGE gene polymorphisms on the development of HCC in chronic HCV patients was analyzed.

In the current study, no-significant association was detected between the four studied groups regarding the heterogeneity in RAGE gene polymorphism. However, the highest frequency of RAGE gene polymorphism was detected in the chronic HCV patients who developed HCC, variant genotype was detected in 83.3 % of patients in contrast to 16.7% who showed wild type of RAGE gene.

These finding agreed with **Shih-Chi et al.** [27] who showed a significant correlation of RAGE gene C/T (rs1800625) polymorphism with liver carcinogenesis, also, it was reported that it has a protective role in the progression of HCC. In consistent with our results, **Shih-Chi et al.** [27] found a significant association between the RAGE gene rs1800625 polymorphism and the increase in risk to HCC when compared to control group.

It has been reported that the rs1800625 C/T polymorphism form of RAGE gene is significantly associated with the development of different types of cancers including, renal, oral [28], lung [28] and liver cancer [29]. In addition to its carcinogenic effect, it has been demonstrated that many RAGE gene polymorphisms are significantly associated with cancer progression [30].

However, previous study failed to demonstrate a significant association between the rs1800625 RAGE

gene polymorphism and increased risk to breast cancer [30]. These cumulative data suggest that many forms of genetic heterogeneity have been implicated in different types of cancers. Although different tumors may share the same risk allele, they have different tumorigenic potential.

RAGE is expressed in hepatic cells, hepatocytes and hepatoma cells [31]. There is accumulating evidence that activation of RAGE signaling pathways in the liver could contribute to the development and progression of numerous types of hepatic disorders [32]. The RAGE signaling pathways have been influenced by numerous RAGE isoforms. Under certain conditions, these signaling pathways are generated by the alternative processing of mRNAs or through a full proteolytic breakdown of the full-length RAGE gene [32].

In order to determine the impact of C/T RAGE (rs1800625) gene polymorphism on the severity of HCC in chronic HCV infected patients, we compared the frequencies of TT and non-TT (TC/CC) genotypes in different risk groups of HCC using log regression analysis. Our results revealed that, there was no-significant difference between both genotypes regarding gender, LN metastases, and BCLC score.

Our results revealed that the heterozygous TC genotype was the most frequent (56 %) in the HCC group when compared with other genotypes (TT&CC). **Shih-Chi et al.** [27], found TT genotype was the most frequent in HCC patients. Moreover, the functional impact of rs1800625 genotype on progression of HCC has been demonstrated in previous studies, they reported that an alternation in RAGE expression positively promotes tumorigenesis in liver cells and negatively regulates the progression of HCC [33].

In the present study, we determined the frequencies of the 63bp deletion polymorphism (-407-345) in the 5' flanking region of RAGE gene among the four studied groups. We detected a high significant difference between the four groups regarding the distribution of 63bp deletion of RAGE gene among them.

The results revealed that the majority of patients who developed HCC "group I" showed non-wild genotype 86.7 % in contrast to 13 % who showed wild type of RAGE gene. Also, we observed higher non-wild genotype in patients with liver cirrhosis "group II" than wild genotype. In agreement with our results, it has

been reported that the 63bp deletion allele (-407-345) is widely associated with different types of malignancies. Moreover, it has been demonstrated that the 63bp deletion variant has a great impact on RAGE expression, therefore, it is significantly implicated in early hepatocarcinogenesis. Therefore, once HCC develops, the RAGE gene expression starts to decline with the increase in tumor growth and progression [34].

In addition, we also explored the potential association between the RAGE gene polymorphisms and the levels of several serum markers of HCC, including a fetoprotein, alanine transaminase, and aspartate transaminase. As a consequence, no significant difference in the serum levels of these markers was detected between patients who possess at least 1 polymorphic allele and those who do not for any of the RAGE SNPs examined

## 5. Conclusion

The current study demonstrated that the RAGE gene polymorphism (63bp deletion) significantly contributed to development of HCC in chronic HCV patients while this contribution was non-significant in rs1800625; however, it is not associated with HCC progression. Accordingly, our data indicated the impact of RAGE gene variations on HCC development in HCV infected patients. Taken together, our results showed that SNP 63bp deletion was frequently distributed among HCC patients which could contribute to an increased risk of HCC. These findings indicate a novel genetic predisposition to liver tumorigenesis.

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