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Research Paper

ASSOCIATION OF *ACE* AND *ACE2* GENES POLYMORPHISMS WITH SUSCEPTIBILITY TO HEPATOCELLULAR CARCINOMA IN EGYPTIAN HCV PATIENTS

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Abstract

Chronic infection with the hepatitis C virus (HCV) is a major risk factor for the development of hepatocellular carcinoma (HCC) worldwide .There is accumulating evidence that the genes controlling the actions of the rennin-angiotensin system (RAS) may influence malignancy development. The objective of the study was to investigate the relation of angiotensin converting enzymes (ACE &ACE2) genes polymorphisms to (HCC) in a group of Egyptian (HCV) patients. The study was conducted on 120 Egyptian HCV and 100 HCC patients along with 130 healthy control volunteers. *ACE* insertion deletion (I/D) gene polymorphism was detected by PCR. ACE2 rs2106809 (C/T) gene Polymorphism was assessed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). HCC patients showed Increased frequency of the *ACE D* allele (high producing allele) compared to HCV patients and control *(p<0.0001).* Increased frequency of *ACE2 T* allele was detected among male HCC patients compared to male HCV patients and control. ***(p< 0.0001)*** .Female HCC patients also showed increased frequency of *ACE2CT* genotype compared to female HCV patients and control***(p<0.001).*** *ACE D* and *ACE2* T alleles may confer a high risk for transition to HCC among Egyptian HCV patients.

Key words: ACE polymorphism , ACE2 polymorphism, HCV, HCC , Liver fibrosis.

INTRODUCTION

Hepatitis C virus (HCV) is a major risk factor for the development of HCC worldwide [1,**2]**. Hepatocellular carcinoma (HCC) is the sixth most common cancer and third leading cause of

cancer related mortality globally[**3].** In Egypt, HCC is a major health problem and its incidence is increasing **[4,5].** Gene therapy is a promising treatment of cancer (64.4% of all gene therapy trials)and the Identification of functionally relevant tumor-specific genes for therapeutic targets remains as the major challenge in cancer gene therapy **[6].**

Variants of genes involved in carcinogenesis may determine an individual s susceptibility to developing HCC. Single nucleotide polymorphisms (SNPs) are the most common type of genomic sequence variation and are thought to be associated with susceptibility to disease and an individual response to treatment[2].There is evidence to indicate that the genes controlling the actions of the rennin- angiotensin system (RAS) may influence cancer development **[7,8]**

Angiotensin converting enzyme (ACE) , a key enzyme in the classical RAS pathway that converts angiotensin I (AngI) to the potent vasoconstrictor angiotensinII (AngII**)** which mediates diverse biological actions through its interaction with AngII type I receptor (AT1) [**9,10].**

Human ACE gene **is** located at 17q23, weighs 21KD and consists of 26 exons. It encodes Angiotensin-II (AT-II). This gene contains a polymorphism based on the presence (insertion [I]) or absence (deletion [D]) of 287-bp alu sequence in intron 16 resulting in three genotypes (deletion/deletion (DD) , Insertion /insertion (II) and deletion /insertion DI).[**11,12**]. Many studies suggest that insertion/deletion (D/I) polymorphism of ACE affects serum level of ACE in the order I I<D I<DD [**12]**

Angiotensin converting enzyme 2 (ACE2) is a recently identified homologue of ACE that degrades AngII to Ang1-7 **[13]**. There **is** evidence that Ang1-7 through binding with its receptor identified as MAS receptor, may modulate the effects of RAS activation *via* s inhibition of ACE and blockade of the AT1 receptor**[14].** Genetic variation in and around the *ACE2* gene which is located on chromosome Xp22 is also a strong candidate for differences in ACE2 activity [**15]**. *ACE2 rs2106809 i*s localized in intron 1, and three SNPS residing upstream of the transcriptional start site of *ACE2* gene at the position of 10.3, 20.4 and 23.2kb, that may affect the *ACE2* gene expression **[16].**

On this basis the aim of the current study was to investigate the association of *ACE* and *ACE2* genes polymorphisms with risk to develop hepatocellular carcinoma in a group of Egyptian HCV patients hoping to identify new functionally relevant HCC-specific genes for therapeutic targets.

MATERIAL AND METHODS:

The present study is an observational case control study, conducted on two groups of patients . Group 1 included 100 patients with HCC attending the outpatient clinics of the Oncology Center-Mansoura University, during the period December 2012 to January 2014. Group 2 included 120 treatment naive, unselected HCV patients attending the outpatient clinics of the internal medicine and the Hepatology unit, , Mansoura university, during the period December 2012 to August 2013. The diagnosis of HCV was confirmed by HCV m RNA quanlitative PCR test. In addition, 130 healthy volunteers were enrolled in this study as the control group. All subjects were genetically unrelated. The size and number of HCCs were defined by triphasic computerized scan or magnetic resonance imaging of the abdomen. The patients were staged and managed according to the Barcelona-Clinic Liver Cancer Group diagnostic and treatment strategy (BCLC) **[17]**

Exclusion criteria were: patients with HBV, history of drug hepatotoxicity, autoimmune liver disease and metabolic liver diseases or having any other cancer.

The study was approved by the ethical committee of Mansoura University , faculty of Medicine, every study participant gave informed consent.

Laboratory analyses:

Specimen collection:

Ten mL blood was withdrawn from each subject enrolled in the study after an overnight fast. Blood samples were aliquoted into 4 tubes. The first aliquot of (5mL) was centrifuged and serum was used for determination of ordinary biochemical analyses. The second aliquot (2 ml)blood collected on EDTA and was stored at -80^{o}C to be used for DNA extraction and assessment of *ACE* (I/D) and *ACE2* rs2106809 (C/T) genes polymorphisms .The third aliquot (1

ml) whole blood used for assessment of complete blood count (CBC) by the automatic counter. For INR determination, 1.8 Ml blood were delivered into citrated tube.

Biochemical analyses:

1- Routine Biochemical analyses

Serum (ALT) and serum (AST) were determined according to the method of **Henry *et al* [18]**and Amador and Wacher**[19**] respectively. Serum albumin was determined according to the method of **Henry *et al.,* [20].** Serum alkaline phosphatase (ALP) was determined according to the method of Bretaudier ***et al.*** **[21]** Serum bilirubin was detected according to the method of Landis and Pardue[**22**] All the former mentioned analyses were performed using kits supplied by Roche diagnostic (GMBHD-68298, Mannheim, Germany).Serum alphafetoprortein (AFP) was detected according to ELISA method **(Hiari *et al*[23]** using kits supplied by BioVendor – Laboratorní medicína USA

DNA extraction:

DNA was extracted from EDTA anticoagulated blood according to the standard procedure using Gentra genomic DNA purification kit.

Determination of *ACE (insertion /deletion) (I/D*) gene polymorphism:

Genotyping of ACEI/D was performed by polymerase chain reaction (PCR) based on DNA amplification using specific flanking primers **[24].**The primer sequences were as follows:

forward primer: 5CTGGAGACCACTCCCATCCTTTCT3

Reverse primer:5GATGTGGCCATCACATTCGTCAGAT3.

The PCR mixture was constituted in a total volume of 25µl that contained 200 ng DNA, 0.2 m M of each of the deoxynucleotide triphosphates; 20nM of each oligonucleotide primer each forward and reverse, reaction buffer (1X), 1.5Mm MgCL2, 0.1U Taq polymerase. The conditions for denaturation, annealing, polymerization and extension were $94^{\circ 0}$C for 5 mins, and 30 cycles of $94^{\circ 0}$C for 30 sec, $64^{\circ 0}$C for 30 sec, $72^{\circ 0\circ}$C for 30 sec and a final extension period of $72^{\circ 0}$C for 5 mins. Amplified PCR products were separated on 2% agarose gel. The presence of 190bp fragments represented the deletion allele (D) allele and the presence of 490 bp fragments represented the insertion allele (I) allele. **(Fig1)**

Detection of *ACE2 rs2106809 (C/T)* gene polymorphism:

The polymorphisms of rs106809 of ACE2 gene was assessed by polymerase chain reaction-restriction fragment length polymorphism (PCR-REFLP) [**25]** .A product Of 207 bp was generated using the

Forward primer 5-GAAAGCCAGATGCTTTAACAAG-3 and the

Reverse primer: 5-TTTTTCCATATCTCTATCTGATCG-3

The resultant PCR products were digested with Taq1 (New England Biolabs, Beverly, MA,USA), resulting in two products of 183bp and 24 bp in the presence of T allele (**Fig2)**

Statistical analysis

Data were analyzed on a personal computer running SPSS© for Windows [16]. Data were summarized as frequencies, mean ±SD, and median (range) whenever applicable. All tests are considered significant if $p \leq 0.05$. The independent-samples t-test was used to compare the means between two groups**.** Comparisons of the genotypic or allelic frequencies between groups were performed using the chi- squares test **.**Odds ratios were calculated with a 95% confidence interval limit.

Sample size calculation:

Using PASS 2008 V 08.0.15;We are planning a study of independent cases and controls with 1 control(s) per case. Prior data from Egypt indicate that the frequency of DD genotype among controls is 0.23. If the true frequency among cases is 0.49, we will need to study 86 case patients and 86 control patients to be able to reject the null hypothesis that the exposure rates for case and controls are equal with probability (power) 0.95. The Type I error probability associated with this test of this null hypothesis is 0.05. We will use an uncorrected chi-squared statistic to evaluate this null hypothesis.

RESULTS:

Clinical characteristics of studied HCV and HCC patients: (Table1).

The study included 100 HCC(23% Females and 77% males) and 120 HCV patients (37%females and 83% male patients. Patients with diabetes mellitus represented 16% of HCC patients and 17.5% of HCV patients .Mild ascites was detected in 24% of HCC patients and 23% of HCV patients, moderate ascites in 18% of HCC patients and 13% of HCV patients. Tense ascites was detected in 10% HCC patients and 10.8% HCV patients. According to Child Pugh score system 32% of HCC patients and 34% of HCV patients were classified in grade A. Grade B represented 55% of HCC patients and 53% of HCV patients. Grade C represented 13% of HCC patients and 12.8% of HCV patients. Regarding HCC patients, single bifocal lesions were detected in 37% of patients. Bifocal and multiple focal lesions were demonstrated in 23% and 40% of patients respectively. Metastasis was detected in 41% of HCC patients. Portal vein invasion was demonstrated in 20% of HCC patients. Serum bilirubin levels and serum AST activity were significantly elevated in HCC patients compared to HCV patients.

Genotypes distribution and alleles frequencies* of *ACE(I/D)gene polymorphism in the studied groups(Table2).

. In the sample population, the relative frequencies of the ACE genotypes were not significantly different from values predicted by Hardy-Weinberg equilibrium.

The frequency of the *ACE I* allele was significantly decreased in HCC patients compared to HCV patients and control, which was31.5% versus 45.8% and 58% for HCV and control respectively. ($p<0.0001$) Odd ratio (OR) 0.5, (95% CI 0.4-0.8) .The frequency of the *ACE D* allele was significantly increased in HCC patients compared to HCV patients and control , which was 68.5% versus 54.2% and 42% for HCV patients and control respectively *($p<0.0001$)*. HCC patients showed increased frequency of ACE DD genotype (49.0%) compared to HCV patients (29.2%) and control (16.9%). ($p<0.0001$). ACE (I/I) genotype was present in low frequency in HCC patients (12%) compared to HCV patients (20.8%) and control (32.2%).

Genotypes and alleles* frequencies of *ACE2* gene in studied groups *(Table3).

In the sample population, the relative frequencies of the ACE-2 genotypes were not significantly different from values predicted by Hardy-Weinberg equilibrium.

The female HCC patients showed increased frequency of ACE2 C/T genotype (56.5%) as compared to female HCV patients (43.2%) and control (48.6%).ACE2CC genotype frequency decreased in female HCC patients (21.7%) compared to control group (35.1%). ACE2 T allele frequency increased significantly in both female HCC (50.0%) and HCV (62.2%) patients compared to control (40.5%) *.($p <0.001$).* OR 0.6; (95% CI 0.3-1.3). *Male HCC patients* showed increased frequency of ACE2 T allele (64%) compared to HCV patients (43.4%) and control (26.8%). *($p< 0.0001$).*

ACE2 C allele frequency decreased significantly in male HCC patients (35.1%) compared to HCV patients (56.6%) and control (73.2%). ($p< 0.0001$).OR 2.4; (95% CI 1.3-4.6)

DISCUSSION:

The role of (HCV) infection as a risk factor for HCC is a subject of intense research [26].To elucidate the possible malignant transition to HCC among HCV patients, we studied the association of ACE and ACE2 gene polymorphisms with susceptibility to HCC in HCV patients.

To the best of our knowledge this is the first study to examine the association of ACE and ACE2 gene polymorphisms as host genetic risk factors for HCC in a group of Egyptian patients with HCV. In this study the first main observation is that HCC patients showed increased frequency of ACE D allele (high producing allele for ACE) This is in agreement with the findings of George et al **[27]**who provided support that mutation in RAS components contribute to the risk of developing certain malignancies. Moreover, in early gastric cancer, the frequency of the I/D and D/D genotypes are increased **[28].**In cancer prostate, The DD genotype and D allele may have statistically significant detrimental effects [29].Furthermore, Du and coworkers demonstrated that moderately increasing AT2R expression could increase the growth of HCC tumors and the proliferation of HCC cells *in vivo* [30]. In contrast to the present study , Yuan and colleagues suggested that D/D genotype of ACE decreased the risk for

HCC [31]. This contradiction may be due to difference in ethnicity of studied population. The Yuan study subjects were all ethnic Han Chinese, and the results should not be extended to other populations. However our results resonates well with the role of the classical RAS pathway " ACE/AngII/AT1 " in the development of liver fibrosis, cirrhosis and HCV- associated HCC. Several studies have shown that AngII could mediate and exacerbate liver fibrosis through hepatic stellate cell activation (HSC) and stimulation of transforming growth factor beta1 (TGFβ-1) secretion via AT1 receptors **[32,33].** In addition, AngII could increase nuclear factor kappa B(NFκB) activity by inhibiting IKb expression in a redox –sensitive manner in HSCs [34]. Moreover, AngII could also increase the expression of activator protein 1(AP1) in HSC cells that increase procollagen m RNA expression via ERK-1/2 pathway in a redox sensitive manner **[35].** Finally, Ang II was shown to stimulate the production of monocyte chemotactic protein 1 (MCP-1) by HSC and modulating hepatic inflammation [36].Besides its role in progression of liver fibrosis, AngII plays a pivotal role in tumor development by different mechanisms. The first is through modulation of tumor angiogenesis by stimulating the expression of several pro-angiogenic agents and growth factors including vascular endothelial growth factor (VEGF), angiopoeitin, basic fibroblast growth factor (b-FGF) and platelet –derived growth factor (PDGF) [7].The second is through its effect on cellular proliferation by increasing the expression of growth –related oncogenes and growth factors **[37].**Finally, AngII can stimulate the release of macrophage/monocyte chemoattractant protein, (MCP-1), and granulocyte colony stimulating factor, thus increasing macrophage infiltration in tumor cells **[38].**

The second main observation in this study is in the ACE2 gene polymorphism which is an X-linked gene and so detected separately among male and female HCC patients. The study demonstrated increased frequency of ACE2rs2106809 T and decreased frequency of ACE2rs2106809C alleles. This may be suggestive for the effect of ACE2rs2106809 T allele effect in down regulating the expression of ACE2 and decreasing Ang1-7 production **[16].**These observations are in agreement with the role of the alternative RAS pathway ACE2/ Ang(1-7)/ MAS receptor axis in attenuating the progression of liver cirrhosis and HCC **[39].** It was documented that ACE2 upregulation may contribute to the attenuation of cirrhosis progression by degrading Ang II. In addition, ACE2 generates Ang1-7, this peptide may reduce AngII mediated vasoconstriction by blocking AT1receptors and attenuating hepatic HSC activation through interfering with AngII induced phosphorylation of ERK1/2 **[11, 28]**. In addition, Ang(1-7) could decrease m RNA expression of connective tissue growth factor and vascular endothelial growth factor, two critical growth factors implicated in fibrosis and tissue repair **[40].** On the other hand, Ang(1-7) plays a pivotal role in attenuating cancer progression through inhibition of angiogenesis as signaling of Ang1-7 through MAS receptor inhibits vascular endothelial growth factor A (VEGFA) expression by repression of ERK signaling32. In contrast to AngII-AT1 , the Ang1-7 MAS- axis is antiproliferative through the repression of ERK signaling **[33].** These observations partly assure that since both ACE2 and ACE coordinate RAS physiological functions. An increase of ACE, together with a decrease of ACE2 activities may confer higher risk for HCC **[41]**.

In summary, there is considerable evidence supporting the concept that opposing axes of the RAS are involved in the pathogenesis of chronic liver diseases. On one side , the ACE/AngII/AT1 receptor axis promotes liver injury and fibrosis progression , on the other, ACE2/Ang(1-7)/ MAS receptor promotes resolution of inflammation .The current study highlights the potentially important role of ACE and ACE2 genes polymorphisms as suspected host genetic markers for prediction of HCC risk among Egyptians HCV patients and functionally relevant HCC-specific genes for therapeutic targets. Further studies are warranted to investigate the therapeutic potential of agents that can modulate the ACE2/Ang1-7/MAS receptor axis of the RAS in HCV and HCC patients

Table (1): clinical characteristics of studied HCC & HCV patients

Variable		HCC		HCV	
		No	*%*	*No*	*%*
Sex	**Male**	77	77.0%	83	69.2%
	Female	23	23.0%	37	30.8%
Diabetes mellitus	**No DM**	84	84.0%	99	82.5%
	DM	16	16.0%	21	17.5%
Ascites	**Absent**	48	48.0%	63	52.5%
	Mild	24	24.0%	28	23.3%
	Moderate	18	18.0%	16	13.3%
	Tense	10	10.0%	13	10.8%
Child Ough score system	**A**	32	32.0%	40	34.2%
	B	55	55.0%	62	53.0%
	C	13	13.0%	15	12.8%
No of lesions	**Single**	37	37.0%	*N/A*	
	Bifocal	23	23.0%		
	Multiple	40	40.0%		
Metastasis	**Absent**	59	59.0%		
	Present	41	41.0%		
Portal vein invasion	**Absent**	80	80.0%		
	Present	20	20.0%		
BCLC	**A**	3	3.0%		
	B	30	30.0%		
	C	47	47.0%		
	D	20	20.0%		
		Mean (SD)	***Range***	***Mean (SD)***	***Range***
Age (years)		60 (10)	28-90	58 (11)	25-90
Albumin(mg/dl)**		3.2 (.8)	1.7-4.2	3.5 (0.9)	2.0-5.0
Bilirubin(mg/dl)		1.9 (1.3)	.0 -7.0	2.3 (2.1	.0-13.0
INR		1.1 (0.3)	1.0-2.1	1.2 (0.4)	1.0-3.0
ALT (U/L)		62 (35)	12-157	60 (38)	12-211
AST (U/L)**		109 (68)	15-312	74 (34)	15-189
WBCs		6.0 (2.4)	2.1-14.1	6.5 (2.9)	2.1-15.1
Hemoglobin g/dl		11.7 (1.9)	5.4-15.2	11.8 (1.9)	5.4-15.2
Platelets		145 (77)	31-314	147 (78)	31-314
AFP (ng/ml)		1213 (2525)	5-15000	N/A	

***The difference was significant between the two groups (t-test); N/A not available*
HCC: Hepatocellular carcinoma
HCV: Hepatitis c virus
INR: international normalized ratio
ALT: Serum Alanine transaminase
AST: Serum Aspartate transaminase
WBCS: White blood cell count
AFP: alphafeto protein
BCLC: Barcelona-clinic liver cancer group diagnostic and treatment strategy

Table (2): Genotypes and alleles frequencies in *ACE* gene

ACE1	Genotype frequencies in ACE1						Chi2	P
	HCC		*HCV*		*Control*			
	No	%	No	%	No	%	31.8	< 0.0001
I/D	39	39.0%	60	50.0%	66	50.8%		
D/D	49	49.0%	35	29.2%	22	16.9%		
I/I	12	12.0%	25	20.8%	42	32.3%		
ACE1	**Allele frequencies in ACE1**						**Chi2**	**p**
	HCC		*HCV*		*Control*			
	No	%	No	%	No	%	31.2	< 0.0001 OR= 0.5; (95% CI 0.4-0.8)
I	63	31.5%	110	45.8%	150	58%		
D	137	68.5%	130	54.2%	110	42%		

HCC: hepatocellular carcinoma
HCV: hepatitis C virus
ACE: Angiotensin Converting enzyme
I/D: insertion /deletion
OR: Odds ratio

Table (3): Genotypes and alleles frequencies in ACE2 gene

ACE2		Genotype frequencies in ACE2						Chi2	p
		HCC		*HCV*		*Control*			
		No	*%*	*No*	*%*	*No*	*%*		
Female	C/T	13	56.5%	16	43.2%	36	48.6%	10.2	0.037
	T/T	5	21.7%	15	40.5%	12	16.2%		
	C/C	5	21.7%	6	16.2%	26	35.1%		
ACE2		**Allele frequencies in ACE2**						**Chi2**	**p**
		HCC		*HCV*		*Control*			
		No	*%*	*No*	*%*	*No*	*%*		
Male	T/0	50	64.9%	36	43.4%	15	26.8%	19.5	0.0001 OR: 2.4(95% CI 1.3-4.6)
	C/0	27	35.1%	47	56.6%	41	73.2%		
Female	T	23	50.0%	46	62.2%	60	40.5%	9.3	0.001 OR:0.6 (95% CI 0.3-1.3)
	C	23	50.0%	28	37.8%	88	59.5%		

HCC: hepatocellular carcinoma
HCV: hepatitis C virus
ACE 2: Angiotensin converting enzyme 2
OR: Odds ratio
ACE1: Odds ratio 0.5; (95% CI 0.4-0.8)
ACE2: Odds ratio in males 2.4; (95% CI 1.3-4.6)
ACE2 Odds ratio in females 0.6; (95% CI 0.3-1.3)

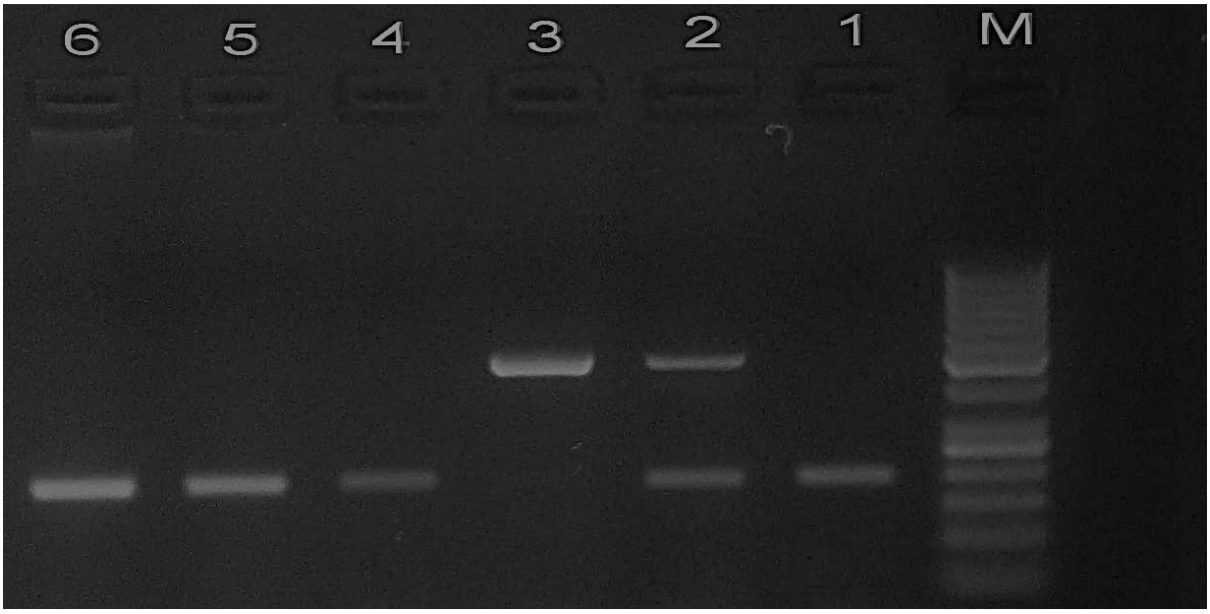

Fig (1): Agarose gel electrophoresis stained with ethidium bromide showing the amplification For ACE (I/D) gene polymorphism using PCR:
M represents the 50 bp lad
Lane 1,4,5,6: Homozygous (DD)
Lane 2 : Heterozygous (ID)
Lane 3 : Homozygous (II)

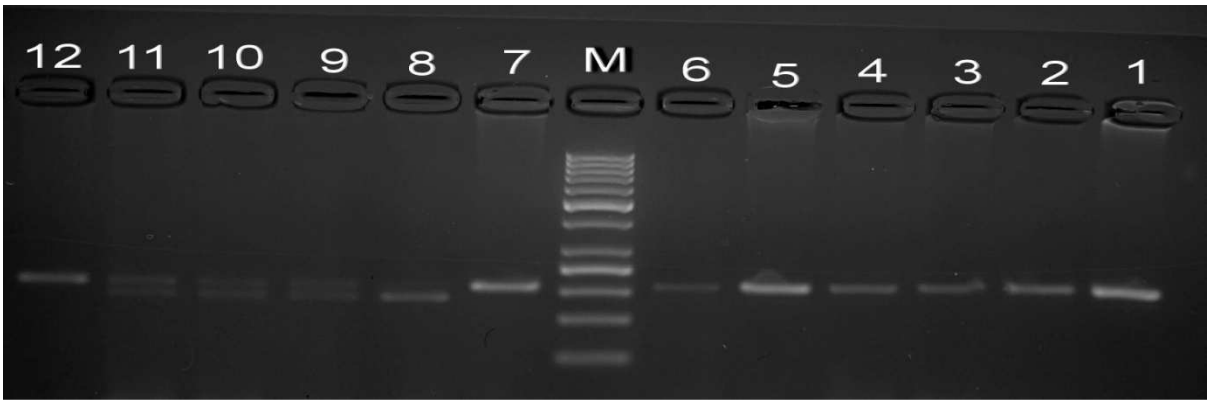

Fig (2): Agarose gel electrophoresis stained with ethidium bromide showing the amplification for polymorphism of ACE2 rs2106809 (C/T) gene using PCR-RFLP:
Lane 1,2,3,4,5,6: PCR product of 207 bp
Lane 7,12 : Homozygous (CC)
Lane 8 : Homozygous (TT)
Lane 9,10,11 : Heterozygous (CT)
M represents the 50 bp ladder

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CONFLICT OF INTEREST
The authors declare no conflict of interest

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