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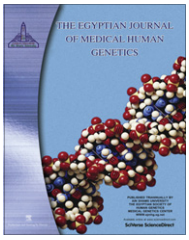

ORIGINAL ARTICLE

Angiotensin converting enzyme (ACE D/I) polymorphism and its relation to liver fibrosis progression in Egyptian patients with chronic hepatitis C virus infection

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KEYWORDS

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Abstract Hepatitis C virus (HCV) infection is a global health problem in Egypt and causes different liver disease spectrum. Evidence indicates that angiotensin I converting enzyme (ACE) gene polymorphism may play a role in determining disease progression. We aimed to determine the association of ACE gene I/D polymorphism with ACE serum levels and to examine the association between different I/D genotypes with the severity of hepatic fibrosis in chronic HCV infected Egyptian patients. Thirty controls and 90 HCV infected patients participated in the study, patients were subgrouped by Ishak stage of fibrosis into subgroup IIa ($n = 30$; fibrosis score 0–1), subgroup IIb ($n = 38$; fibrosis score 2–3) and subgroup IIc ($n = 22$; fibrosis score 4–6). DNA was multiplied by polymerase chain reaction (PCR). ACE genotype frequency in HCV infected patients was significantly different comparing to controls ($X^2 = 7.169$, $P = 0.028$). With non-significant difference in ACE D/I genotypes and allele frequencies among the patient subgroups ($P > 0.05$),the frequency of the DD, DI and II genotypes in subgroup IIa, subgroup IIb and subgroup IIc were (53.3%, 36.6%, 10%), (44.7%, 44.7%, 10.5%) and (50%, 22.7%, 27%), respectively. The D and I allele frequency were (71.66%, 28.33%), (67.1%, 32.9%) and (61.36%, 38.63%), respectively. ACE serum levels were significantly increased in DD more than DI and II ($t = 2.56, 3.43$, $P < 0.05$), respectively, with non-significant

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association in sonographic findings, viral load and liver function test (LFT) parameters with the ACE genotypes. Serum ACE levels were significantly increased in all patient subgroups when compared to controls with a non significant difference of ACE levels between subgroup IIb and IIc. We concluded that the D/D genotype is associated with HCV infection but not associated with degree or the progression of hepatic fibrosis.

1. Introduction

The natural history of the chronic liver disease caused by HCV remains controversial with varying rates of progression to cirrhosis [1]. Although approximately 80% of patients who acquire hepatitis C virus (HCV) infection will develop a chronic low grade slowly progressive hepatitis, perhaps only 20–30% of infected patients will progress to clinically significant fibrotic disease after 20–30 years [1]. Hepatic stellate cells (HSCs) – the main collagen-producing cells in the liver, are playing an essential role in fibrogenesis process [2]. Multiple animal studies suggest that angiotensin II (AT-II) contributes to hepatic fibrosis. This is primarily due to induction of profibrotic pathways through the AT-II receptor type 1 (AT1 receptor) by AT-II, which promotes the activation of the HSCs [3,4]. Multiple animal model studies have demonstrated that AT-II-blocking agents, such as angiotensin-converting enzyme (ACE) inhibitors and AT-II type 1 receptor antagonist (AT1A), attenuate liver fibrosis [5]. Experimental animal studies on mice have demonstrated that AT_{1A} receptor-deficient mice are protected from hepatic fibrosis whereas AT_2 receptor-deficient mice have worse fibrosis [6–8].

The renin–angiotensin system (RAS) is a hormone system that regulates blood pressure and water balance [9]. It has been said to be involved in the pathogenesis of several diseases including fibrosis in the liver, kidney, heart and lung during chronic inflammation through the regulation of cell growth, inflammation, oxidative stress and fibrosis [9]. The ACE gene insertion/deletion (I/D) polymorphism was first identified in 1990. The gene encoding ACE is located on chromosome 17q35 and consists of 26 exons. A 250-bp deletion/insertion polymorphism exists in intron 16 of the ACE gene and the deletion variant is associated with higher serum levels of the enzyme [10]. The ACE is suggested to be the key enzyme in the RAS system through converting AT-I to the potent vasoconstrictor AT-II [11]. The role of the ACE gene I/D polymorphism as a risk factor has been investigated in several diseases [12]. The findings that ACE inhibitors and AT-II receptor antagonists decrease hepatic fibrosis support the effects of angiotensin and its receptors on liver fibrosis [13]. It was found that serum ACE levels were found to be increased approximately two times in people with DD allele of ACE gene [14].

Certain host and environmental factors affecting the development of fibrosis in chronic HCV infection have been identified such as male gender, excess alcohol consumption, age of acquisition of infection, and obesity [15,16]. However despite this, it remains difficult to assess an individual's risk of developing progressive fibrotic disease. Such prognostic uncertainty and variation in the response to a common infective agent suggest a genetic component to the progression of fibrosis in HCV infection [17].

2. Aim of the study

The current study aimed at determining the association of ACE gene I/D polymorphism with ACE serum levels and whether there is a significant association between different I/D genotypes with the severity of hepatic fibrosis in chronic HCV infected Egyptian patients.

3. Subjects and methods

This study was carried out in the Medical Biochemistry and Internal Medicine, Pathology Departments, Faculty of Medicine, Outpatients Clinics and Intensive Care Unit of Zagazig University Hospitals during the period from June 2010 to November 2011. In this study we investigated the effect and relationship of allelic variants of the ACE gene in Egyptian patients with chronic HCV infection with different degree of liver fibrosis according to Ishak classification [18]. PCR technique with restriction fragment length polymorphism (RFLP) was used to detect gene polymorphism.

4. Subjects

One hundred and twenty subjects were included in this study. They were classified into two main groups:

4.1. Group I

Thirty healthy control volunteers (17 females, 13 males) with mean ages ± S.D. of 46.76 ± 6.82 years. They are apparently healthy volunteers not complaining of chronic diseases. Exclusion criteria were positive viral markers (such as hepatitis B and C), history of alcohol use, diagnosis of autoimmune hepatitis (a non-resolving inflammation of the liver of unknown cause, characterized by the presence of interface hepatitis on histologic examination, hypergammaglobulinemia, and autoantibodies) [19], or metabolic liver disease (hemochromatosis, Wilson's disease, non alcoholic steatohepatitis) [20] liver disease associated with drug use and primary biliary cirrhosis that can be diagnosed by symptoms as a general feeling of tiredness, fatigue, pruritus, dry eyes and mouth and jaundice or can be diagnosed by laboratory findings as anti-mitochondrial antibody (AMA) blood test, abnormal liver function test (elevated gamma-glutamyl transferase (GGT) and alkaline phosphatase (ALP), and abdominal ultrasound that shows whether the liver and bile ducts are inflamed [21].

4.2. Group II "chronic HCV infection"

This group included 90 patients (43 females, 47 males) with mean ages ± S.D. of 49.25 ± 6.7 years. They did not receive any treatment of hepatitis C virus before the study. They

Table 1 Comparison of clinical and laboratory data of the all studied groups (mean ± S.D. and range, with Anova test).

	Group I $N = 30$	Subgroup IIa $N = 30$	Subgroup IIb $N = 38$	Subgroup IIc $N = 22$	F value P value
Sex	−17 Females	−16 Females	−18 Females	−9 Females	$F = 0.489$
	−13 Males	−14 Males	−20 Males	−13 Males	$P = 0.690$
Age (years)Range	46.76 ± 6.82	48.1 ± 6.11	49.07 ± 7.15	51.13 ± 6.96	$F = 1.94$
	37.0–58.0	39.0–59.0	40.00–62.0	38.00–63.0	$P = 0.126$
Serum ACE (U/L)	64.65 ± 16.85	112.86 ± 15.61	180.77 ± 36.57	185.95 ± 21.44	$F = 155.69$
	39.50–98.0	69.70–143.00	110.70–243.60	133.7–218.5	$P = 0.000$
Serum ALT (IU/L)	23.9 ± 6.48	89.8 ± 22.01	131.71 ± 36.58	120.81 ± 30.97	$F = 99.0$
	15.00–34.0	45.0–123.0	76.0–254.0	76.0–189.0	$P = 0.000$
Serum AST (IU/L)	23.23 ± 5.26	108.93 ± 34.08	138.71 ± 38.22	140.0 ± 34.05	$F = 93.13$
	13.0–33.0	56.0–199.0	75.0–200.0	87.0–199.0	$P = 0.000$
Serum ALP (IU/L)	25.33 ± 7.52	34.40 ± 9.37	32.55 ± 8.88	33.63 ± 7.09	$F = 7.26$
	15.0–40.0	22.0–56.00	20.0–51.0	23.0–51.0	$P = 0.000$
Serum albumin g/dl	4.04 ± 0.55	3.04 ± 0.58	2.96 ± 0.64	2.89 ± 0.64	$F = 23.54$
	3.2–5.2	2.2–4.2	2.00–4.2	2.00–4.00	$P = 0.000$
T.bilirubin mg/dl	0.96 ± 0.33	1.95 ± 0.42	1.89 ± 0.40	1.92 ± 0.41	$F = 43.35$
	0.30–1.60	1.20–2.90	1.0–2.70	1.10–2.87	$P = 0.000$
Viral load Eq/ml	–	350×10^3–2×10^6	950×10^3–555×10^4	55×10^5–2×10^7	$F = 106.276$
		1×10^6 ± 416.720	2970×10^3 ± 1.319.142	10.300×10^3 ± 4.464.734	$P = 0.000$

were diagnosed by clinical, laboratory and ultrasonography as HCV infected patients. HCV infection was diagnosed as +ve ELISA for anti HCV antibodies. The presence of anti-HCV antibodies was determined in serum samples by enzyme linked immunosorbent assay technique (ELISA) and +ve PCR for HCV-RNA tested by reverse transcription polymerase chain reaction (RT-PCR) performed on patient sera using Amplicor (Roche Diagnostics, Lewes, UK). All liver biopsy specimens were taken under guided ultrasonography and graded for the degree of necro-inflammatory activity and staged for the extent of fibrosis according to the criteria of Ishak classification [18]. History of risk factors for HCV infection and alcohol consumption were established. Duration of infection at the time of liver biopsy was also recorded.

The study was approved by Zagazig University Ethics Committee. Informed consent was obtained from all patients before participation in this study. The characteristics of both control and studied patient subgroups are listed in Table 1.

5. Sample collection

Ten milliliters fasting venous blood samples were collected from the subjects using standardized protocol and equipment, separated into two samples one whole blood for DNA extraction and ACE gene polymorphism determination and the other serum sample for estimation of ACE levels and other liver function test (LFT) parameters. They were measured by standard chemical and enzymatic commercial methods in the Medical Biochemistry Department, Zagazig University.

5.1. Methods

All subjects are submitted to the followings:

(1) Full medical history taking and complete clinical examination.
(2) Abdominal ultrasonography examination with evaluation of different hepatobiliary parameters including portal, hepatic and mesenteric vein diameters.
(3) Liver biopsy for patients with chronic HCV infection.
(4) Laboratory investigations including:

(A) Routine investigations:

(*) Liver function tests:

- Serum alanine transaminase (ALT), aspartate transaminase (AST) and alkaline phosphatase (ALP) using bio-Merieux kit [22].
- Serum total bilirubin levels [23].
- Serum albumin using photometric colorimetric test [24].

(∗) Viral hepatitis markers: HCV antibodies by ELISA, HCV RNA–RT-PCR and HBs Ag and another hepatotropic virus (A).

- Hepatitis biopsy specimen should contain four pieces of information to most completely assess the specimen and serve patient care [18]:

(1) The statement that it is, indeed, chronic hepatitis.
(2) The grade of activity (including the name of the scoring system used).
(3) The stage of activity (including the name of the scoring system used).
(4) The known or suspected cause of the hepatitis.
(5) Hepatitis related changes (hepatitis C-related fat, increased iron uptake, large cell and small cell changes) and particularly common concomitant diseases, such as alcoholic and nonalcoholic fatty liver disease and hemochromatosis.

6. Laboratory analysis

- Estimation of serum ACE levels using spectrophotometric assay [25].
- ACE gene polymorphism by PCR technique [26,27].

6.1. ACE gene I/D genotyping

Genomic DNA to be used in molecular analysis was isolated and prepared from leucocytes using Biospin Blood Genomic DNA Mini-prep Kit (Sigma, Aldrich Co. LLC, NA2000).

The analysis of I/D polymorphism that is located in 16th intron of ACE gene, was performed with PCR. The PCR was performed in a Perkin Elmer 4800 thermal cycler (PTC-100 machine, MJ Research, Inc., Watertown, Mass. USA). For each PCR reaction the reaction mix prepared as the end volume had to be 25 µl by using 10 pmol/µL.

F 5′CTGGAGACCACTCCCATCCTTTCT3′ and R5′GA TGTGGCCATCACATTCGTCAGAT3′ primers, contained 4 dNTP (Roche-Almanya), 10XPCR tamponade (100 mM Tris–HCl, 15 mM $MgCl_2$, 500 mM KCl, pH: 8.3) (Roche-Almanya), 1.25U Taq DNA Polymerase and 100 ng genomic DNA with a concentration of 30 µmol/ml. Reaction was performed in 35 cycles each composed of denaturation in 95 °C for 5 min, annealing in 94 °C for 30 s and extension in 69 °C for 45 s. After DNA amplification, PCR products were taken on 2% agarose gel electrophoresis and analyzed under UV transilluminator (Illuminyx UVB Transilluminator, USA) by staining 0.5 µg/ml ethidium bromide. Running conditions were 70 V, 100 mA and140 min. 100 Base-Pair Ladder (Bioron) was 0.2 mg/ml in 10 Mm Tris (pH 8.0), 1 mM EDTA. Sub-marine gel electrophoresis was used (Pharmacia Biotech by SEMKO AB, Sweden) using submarine chamber (Maxicell, EC 360 M-E-C apparatus Cooperation ST. Petersburg. Florida, USA). PCR method resulted in a 190 bp product that showed deletion (D allele) and in the presence of insertion, produced a 490 bp product (I allele). In heterozygous samples, two bands of 490 and 190 bp were detected [26,27].

6.2. Statistical analysis

Results were statistically analyzed using SPSS version 11.5 for Windows. The statistical data were calculated for mean and standard deviation (S.D.). Analysis of variance (ANOVA) was used to compare the results of all examined cases in all studied groups within group comparisons. The differences between mean values for each element were tested by student's "*t*" test. The Hardy–Weinberg equilibrium or odds ratio (OR) and 95% confidence interval (CI) for the presence of HCV infection and different liver fibrosis grades within the ACE genotypes were analyzed by using the chi square χ^2 test. Results were considered significant when $P < 0.05$ [25,28].

7. Results

Ninety chronic HCV infected patients (43 females, 47 males with mean ages of 49.25 ± 6.7 years) and 30 controls (13 males, 17 females with mean ages of 46.76 ± 6.82 years) were evaluated in this study. Anova test (*F*-test) revealed a non-significant difference between groups regarding sex and age ($F = 0.489$, $P = 0.690$ and $F = 1.94$, $P = 0.126$), respectively as illustrated in Table 1.

7.1. Fibrosis scores (Fig. 1)

- Ishak classification subgrouped the group II patients according to the stage of liver fibrosis on liver biopsy into three subgroups as follows (Fig. 1).
- Subgroup IIa "HCV with fibrosis score 0 or 1": This subgroup comprised 30 patients (33.33%) with ages ranged between 39.0 and 59.0 years with a mean value ± S.D. of 48.1 ± 6.11 years (16 females and 14 males).

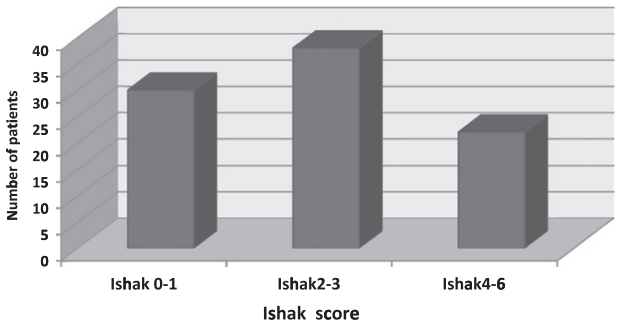

Figure 1 Number of group II patients according to Ishak classification.

- Subgroup IIb: "HCV with fibrosis score 2 or 3": This subgroup included 38 patients (42.22%) (18females and 20 males) with ages ranged from 40.00 to 62.0 years with a mean value ± S.D. of 49.07 ± 7.15 years.
- Subgroup IIc: "HCV with fibrosis score 4 or 6": This subgroup comprised 22 patients (24.44%) (9 females and 13 males) with ages ranged between 38.00 and 63.0 years with a mean value ± S.D. of 51.13 ± 6.96 years.

There was no statistical difference between all groups regarding age and sex ($P > 0.05$).

7.2. Distribution of polymorphism of ACE gene

The frequency of ACE I/D genotypes is summarized in Table 2 and Fig. 2.

In controls and patients with chronic HCV infection (group II), the frequency of D and I allele was 33 (55%) and 27 (45%) and 121 (67.22%) and 59 (32.78%), respectively. The frequency of D allele was more frequent in group II patients but this increase was non-significant when compared to controls ($X^2 = 2.924$, $P = 0.061$ with OR = 0.596 with 95% CI of 0.328–1.082). The DD, DI and II genotypes were recorded in controls and group II patients as follows 7 (23.33%), 19 (63.33%), 4 (13.33%) and 44 (48.8%), 33 (36.6%) and 13 (14.4%, respectively. However, there was a significant association of genotype frequency in HCV infected patients (group II) when compared to the controls ($X^2 = 7.169$, $P = 0.028$), we referred this non-significant result in D and I allele frequency between control and group II to the relatively small sample of the current study.

However, results recorded a non-significant difference in ACE D/I genotypes and allele frequencies between the chronic HCV patient subgroups (Tables 2 and 3).

– In subgroup IIa: The homozygous DD was detected in 16 patients (53.3%), the heterozygous DI in 11 patients (36.6%) and the homozygous II in 3 patients (10%). The D and I allele frequency were 71.66% and 28.33%, respectively.
– In subgroup IIb: There were 17 patients (44.7%) of homozygous DD genotype and there were similar number of patients with DI genotype, we found only 4 (10.5%) patients carrying the homozygous II genotype. The D and I allele frequency were 67.1% and 32.9%, respectively.

Table 2 Allele and genotype frequencies for ACE polymorphism in all studied groups.

Groups	Genotype frequency *n* (%)			Allele frequency *n* (%)	
	DD	DI	II	D	I
Group I	7 (23.33%)	19 (63.33%)	4 (13.33%)	33 (55%)	27 (45%)
Group II	44 (48.8%)	33 (36.6%)	13 (14.4%)	121 (67.22%)	59 (32.78%)
Subgroup IIa	16 (53.3%)	11 (36.6%)	3 (10%)	43 (71.66%)	17 (28.33%)
Subgroup IIb	17 (44.7%)	17 (44.7%)	4 (10.5%)	51 (67.1%)	25 (32.9%)
Subgroup IIc	11 (50%)	5 (22.7%)	6 (27%)	27 (61.36%)	17 (38.63%)

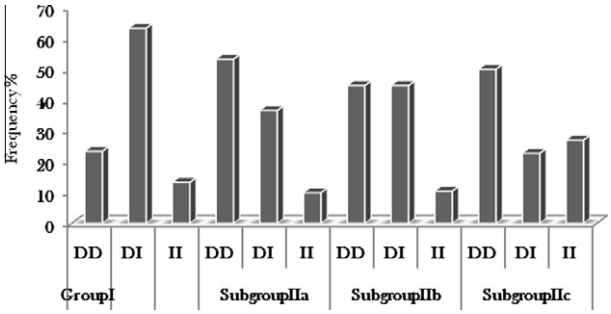

Figure 2 Frequency of different ACE genotypes in all studied groups.

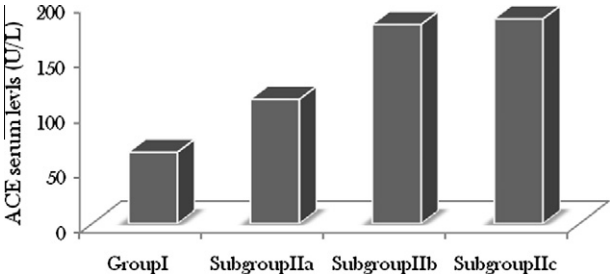

Figure 3 Mean of serum ACE levels in ACE genotypes in all studied groups.

– In subgroup IIc: The ACE homozygous DD, heterozygous DI and homozygous II genotypes were detected in 11 patients with chronic HCV infection (50%), 5 patients (22.7%) and 6 patients (27%), respectively. The D and I allele frequency were 61.36% and 38.63%, respectively.

We revealed a non-significant association with ACE gene (I/D) polymorphism among chronic HCV infected patients ($P > 0.05$), the distribution of ACE genotype was similar between subgroups as illustrated in Table 2.

Serum ACE and the viral load mean ± S.D. levels related to different groups are listed in Table 1. The different genotypes in each group showed different means ± S.D. of ACE serum levels (Fig. 3), however the ultrasonographic findings [right liver (cm), superior mesenteric vein diameter (mm) and portal vein diameter (mm)] and viral load values (Eq/ml) recorded nonsignificant differences among different ACE D/I genotypes of group II (HCV infected patients) listed in Table 4.

ANOVA test (*F*-test) revealed a significant difference of the mean values of serum ACE level and viral load values among different studied groups ($F = 155.69$ and $F = 106.276$, $P = 0.000$), respectively. Serum ACE levels were significantly higher in patients with chronic HCV infection than controls ($t = 13.816$, $P = 0.000$). On comparing serum ACE levels among HCV infected patient subgroups, their levels were significantly increased in subgroup IIb and subgroup IIc more than subgroup IIa ($t = 11.01$, $t = 13.05$, $P = 0.00$), respectively, with non-significant difference between two subgroups IIb and IIc ($t = 1.286$, $P = 0.212$).

Viral load values recorded significant increase in subgroup IIc and subgroup IIb patients more when compared to subgroup IIa with significant increase in subgroup IIc more than subgroup IIb patients ($t = 9.738$, $t = 8.109$ and $t = 7.637$, $P = 0.000$), respectively.

Data of Table 4 revealed that there was a significant increase in the serum ACE levels in control subjects and patients with chronic HCV infection carrying the DD genotype compared to subjects of DI and II genotypes in all studied groups ($P < 0.05$).

7.3. Data of liver function test (LFT) is listed in Table 1

Regarding serum ALT, AST levels there was a significant increase in group II patients with chronic HCV infection when compared to controls ($t = 16.96$, $t = 13.29$, $P = 0.000$), respectively.

Table 3 Chi square and OR (95% CI) of ACE genotypes and allele frequency in all studied groups.

Groups	ACE genotype	D and I allele frequency	OR of D and I allele frequency 95% CI
Control group vs group II	$X^2 = 7.169$	$X^2 = 2.924$	0.596
	$P = 0.028$	$P = 0.061$ (0.05)	0.328–1.082
Subgroup IIa vs subgroup IIb	$X^2 = 0.525$	$X^2 = 0.327$	1.240
	$P = 0.769$ (NS)	$P = 0.582$ (NS)	0.593–2.593
Subgroup IIa vs subgroup IIc	$X^2 = 3.01$	$X^2 = 1.22$	1.59
	$P = 0.22$ (NS)	$P = 0.296$ (NS)	0.697–3.641
Subgroup IIb vs subgroup IIc	$X^2 = 4.26$	$X^2 = 0.404$	1.284
	$P = 0.118$ (NS)	$P = 0.556$ (NS)	0.593–2.782

Table 4 Serum ACE levels (U/L), ultrasonographic and viral load (Eq/ml) of different genotypes in all studied groups.

Groups	DD $n = 7$	DI $n = 19$	II $n = 4$	t-Value	P value
Group I (controls) ACE range (U/L)	76.0–98.0	43.0–77.40	39.50–48.60	4.248*	$P = 0.005$
Mean ± S.D.	88.02 ± 8.22	60.47 ± 10.14	43.57 ± 3.92	9.288**	$P = 0.003$
Group II (HCV patients)	DD	DI	II	2.558*	$P = 0.015$
	$n = 44$	$n = 33$	$n = 13$	3.43**	$P = 0.005$
ACE range (U/L)	110.0–243.60	100.80–234.0	69.70–133.70		
Mean ± S.D.	178.24 ± 9.70	153.6 ± 36.81	108.76 ± 20.03		
Group II: liver right lobe (cm) range: mean ± S.D.	12.8–18.8	12.5–19.2	13.7–18.4	−0.611*	$P = 0.546$
	15.73 ± 1.55	15.77 ± 1.58	15.86 ± 1.65	0.401**	$P = 0.696$
				−0.342***	$P = 0.738$
Group II: superior mesenteric vein diameter (mm) range: mean ± S.D.	7.5–14.6	8.9–13.7	9.6–13.6	−1.870*	$P = 0.071$
	10.44 ± 1.74	11.22 ± 1.33	10.92 ± 1.23	−0.405**	$P = 0.692$
				−0.201***	$P = 0.844$
Group II: portal vein diameter (mm) range	10.1–17.8	10.1–18.1	13.7–17.6	−0.745*	$P = 0.462$
Mean ± S.D.	14.65 ± 2.17	14.82 ± 2.01	15.77 ± 1.33	−2.044**	$P = 0.063$
				−1.87***	$P = 0.085$
Group II: viral load range: mean ± S.D. (Eq/ml)	380×10^3–20×10^6	350×10^3–20×10^6	500×10^3–10×10^6	−1.226*	$P = 0.229$
	3340×10^3 ± 368.706	4340×10^3 ± 515.983	5840×10^3 ± 390.342	−0.172**	$P = 0.866$
				0.951***	$P = 0.361$

* Referred to t-value of comparing DD with DI.
** t-Value of comparing DD with II.
*** t-Value of comparing DI with II ACE genotypes.

Table 5 Laboratory parameter levels and Anova (*F*-test) with different ACE genotypes in all studied groups.

	DD $N = 51$	DI $N = 52$	II $N = 17$	F value P value
Serum ALT levels (IU/L)	102.86 ± 45.73 15.00–254.0	80.19 ± 54.99 15.0–199.0	97.52 ± 43.11 17.0–200.0	$F = 2.793$ $P = 0.065$
Serum AST (IU/L)	103.27 ± 56.82 13.0–200.0	102.17 ± 61.71 16.0–200.0	97.58 ± 33.71 19.0–134.0	$F = 0.065$ $P = 0.937$
Serum ALP (IU/L)	32.46 ± 8.01 20.0–55.0	30.98 ± 9.33 15.0–51.00	29.88 ± 11.15 16.0–56.0	$F = 0.353$ $P = 0.703$
Serum albumin (g/dl)	3.18 ± 0.73 2.0–5.2	3.16 ± 0.71 2.0–4.0	3.65 ± 0.89 2.00–5.10	$F = 2.67$ $P = 0.065$
Serum T. bilirubin (mg/dl)	1.59 ± 0.64 0.30–2.90	1.81 ± 0.46 1.00–2.87	1.55 ± 0.63 0.30–2.30	$F = 2.43$ $P = 0.092$

On comparing ALT and AST serum levels among group II subgroups, there was a significant increase in subgroup IIb and subgroup IIc than subgroup IIa patients ($t = 5.123$, $t = 3.371$, $t = 2.641$, $t = 2.117$, $P < 0.05$), respectively, with non-significant difference between subgroup IIb and subgroup IIc ($t = -0.88$, $t = -0.131$, $P > 0.05$).

Serum ALP and T. bilirubin levels were significantly increased in group II patients than controls ($t = 4.30$, $t = 9.12$, $P = 0.000$), respectively, with non-significant difference among all patient subgroups ($P > 0.05$). Serum albumin levels showed significant decrease in group II patients than controls ($t = -6.28$, $P = 0.000$), with non-significant change among patient subgroups ($P > 0.05$). Table 5 illustrated that there was a non-significant association between all LFT parameters and the genotype pattern of the ACE gene ($P > 0.05$).

8. Discussion

Egypt has the largest epidemic of HCV in the world. The released Egyptian Demographic Health Survey revealed that the percentage of HCV infection in Egypt was 14.7% [29]. Hepatitis C was generally found to be worse, both with greater severity of activity and more rapidly advancing fibrosis [30]. Subsequent to successful long-term maintenance of immune competence, this expectation of worse or more rapid injury is not necessarily reflected by the biopsy specimens that now come from patients with coinfection [31].

Hepatic stellate cells (HSC) are thought to play a pivotal role in fibrogenesis within the liver, and there is a large body of evidence to support the hypothesis that Angiotensin II (Ang-II) promotes activation and dedifferentiation of these cells into myofibroblasts [32]. Furthermore, Ang II encourages myofibroblast contraction, proliferation and promotes release of inflammatory cytokines as well as the deposition of extracellular matrix (ECM) [33]. Although both of the Ang II receptors (AT_1 and AT_2) are expressed in the liver, the AT_1 receptor is far in abundance and is thought to be responsible for most of the Ang II-mediated effects [34]. Multiple studies have shown that angiotensin-receptor blockade leads to downregulation of pro-inflammatory/profibrotic cytokines and postulated that inhibition of angiotensin II decreases the generation of reactive oxygen species, resulting in less collagen synthesis by HSCs, and downregulates the vascular endothelial growth factor, thus attenuating fibrosis [35]. Angiotensin-1 A (AT1A) receptor knockout mice have demonstrated reduced liver fibrosis compared with wild-type mice in a carbon tetrachloride model [36].

A great deal of evidence supporting the role of the renin–angiotensin system (RAS) in hepatic fibrosis has come from animal studies using ACE inhibitors and angiotensin receptor blockers (ARB). Numerous studies using a variety of animal models have demonstrated antifibrotic effects of these drugs [36,37].

These researches' results and evidences of the fundamental role of RAS in the regulation of hepatic fibrosis guided us to examine the possible effect of D/I SNP of ACE gene as a component of RAS gene upon fibrosis in chronic HCV infection among Egyptians. In our present study, patients with chronic HCV infection showed a significant increase in DD genotype when compared to the controls, the frequency of D allele was increased in group II patients but this increase was not significant, we speculated this non-significance may be referred to the small number of our patient sample. Moreover, a non-significant association was determined between DD, DI and II genotype frequency of ACE gene and fibrosis stage among patients with chronic HCV infection ($P > 0.05$).

These findings were in concordance with Mohamed et al. [38], who recorded significant higher percentage of HCV Egyptian patients having the I/D and DD genotypes than the healthy controls.

Forrest et al. [39] studied different four polymorphisms of RAS gene and could not identify any significant association between these four RAS polymorphisms and fibrosis in chronic HCV infection. In addition another study of Güçlü et al. [40] suggested that ACE gene had no role in the development of fibrosis in non-alcoholic fatty liver disease.

Serejo et al. [41] recorded that DD genotype was significantly more prevalent among chronic hepatitis C (CHC) patients but in contrast to our results they declared that ACE I allele may be a risk factor for liver fibrosis progression.

Our findings also revealed a significant association between the increase in the frequency of D allele of ACE gene and ACE serum levels, patients carrying the DD genotype were having a significant increase in serum ACE levels ($P < 0.05$) but this genotype have a non-significant association with each of ultrasonographic findings, viral load values and other LFT parameters ($P > 0.05$).

In concert with our results, Samani et al. [42], Baudin [43] and Gunes et al. [44] have found that plasma ACE levels were

higher in ACE DD carriers than ID and II with hypertension and myocardial infarction patients, respectively.

In contrast to our results, Powell et al. [45] and Forrest et al. [39] reported a non-significant correlation between ACE activity and ACE genotype in chronic HCV. Moreover, Bülent et al. [46] failed to find any significant association between serum ACE levels and ACE gene I/D polymorphism genotypes in patients with Osteoarthritis.

We reported a significant increase in serum ACE levels and other LFT parameters in chronic HCV infected patients compared to controls but we failed to record any significant difference among different fibrosis stages in HCV infected patient subgroups except for viral load values which recorded a significant increase in subgroup IIc and subgroup IIb more than subgroup IIa patients meaning that the viral load may play a pivotal role in hepatic fibrosis progression. Previous studies have also detected increased serum ACE levels in patients with chronic liver disease, but they have suggested that ACE activity is an effective discriminator of the severity of liver disease [47,48]. This was not matched with our results as we could not find any significant association between ACE genotypes' distribution and the different fibrosis stages in patients with chronic HCV infection and even the serum ACE levels did not show any significant differences in patient subgroups.

Meaning that we could not rely on ACE D/I gene polymorphism, ACE serum levels or other liver function test parameters to determine the definitive stage of fibrosis or suspect the course or the severity of the disease in HCV infected patients. But these observations cannot mask the potential importance of RAS in the hepatic fibrogenesis. However, it does suggest that whatever be the mechanism of the increased expression of the ACE levels in DD genotype carrier in chronic HCV infection but that does not suspect the severity and the course of fibrotic liver disease in HCV infection.

These conflicting and opposing results may be explained by the several genetic polymorphisms which exist in the RAS and some of these are recognized to account for the inter-individual variation in RAS activity [39].

However, the pathological risk of ACE DD genotypes also varies between populations with different genetic and environmental backgrounds, suggesting that the ACE DD genotype is acting as a disease modifier rather than as a disease susceptibility factor [43].

Different studies show that AT-II increased liver fibrosis via HSCs, which are activated in damaged liver and a target for AT-II by expressing AT-I receptors in cell surface, while AT-II had no effect on normal healthy liver [32,33,34].

Bulent et al. [47] recorded an increase in the frequency of DD genotype in individuals with fatty liver causing higher levels of serum AT-II levels but no association with necro-inflammation or the level of liver fibrosis suggesting that high frequency of AT-II related with DD genotype prepared a background for disease progression in association with fattening which is the first move and that it is not responsible for direct progression.

Several genetic polymorphisms in the RAS exist and some of these are recognized to account for inter-individual variation in RAS activity and this mechanism might account for the variable rate of progression observed in chronic HCV infection [39,49,50,51].

In conclusion, we found an association between ACE D/I gene polymorphism, DD genotype and ACE serum levels with HCV infection but we have been unable to identify any association between ACE genotype and the development or the progression of fibrosis in chronic HCV infection. The frequency of D/D genotype was higher in chronic HCV patient group but it did not have any effect on necro-inflammatory activity and fibrosis stage. However, it should be noted that these conflicting results of our study and other different studies are referred to relatively small sample studies and the absence of hepatitis C virus genotype as the results of this study indicate that HCV genotype 4 in Egypt is extremely variable, not only in terms of sequence, but also in terms of functional and immunological determinants [52,53]. Adequate larger sample studies must be undertaken for more explanation of the suspected role of ACE gene polymorphism and different RAS component polymorphisms should be examined in the development and progression of fibrosis in HCV to solve this issue. It seems that other factors such as environmental factors and viral load may play a more significant role in this process.

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