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Original Research Article

Apolipoprotein-E Gene Polymorphism and Possible role of *ApoE* ε4 Allele with a Lower Probability of Progression to HCV-Related Liver Cirrhosis in Egyptian Patients

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Abstract

Apolipoprotein E *(ApoE)* plays an important role in regulating lipid and lipoprotein metabolism and *ApoE* genotypes are known to affect lipoprotein concentrations. *ApoE* may become a major variable of preventive and personalized medicine due to several biological role. *ApoE* genotypes could be an important host genetic factor affecting disease progression in chronic liver disease. We investigated whether *ApoE* gene polymorphism determines the disease progression to liver cirrhosis in hepatitis C virus (HCV)-infected Egyptian individuals. This case-controlled study enrolled 120 subjects, 80 chronic hepatitis C (CHC) related liver disease patients and 40 age and sex matched healthy control subjects. *ApoE* genotypes were determined by Restriction Fragment Length Polymorphism (RFLP). Restriction isotyping using restriction enzyme (Hhal). Among the 120 subjects, the most common genotype was ε3/ ε3, accounting for (91.67)%, followed by ε3/ ε4 (8.33) %. The genotypes of ε2/2 2/3 2/4 and ε4/4 were not detected in our results. The ε3 allele was the most common allele overrepresented in CHC LC/LF (98.75) % versus (93.75) % in non cirrhotic group. However, CHC non cirrhotic patients had a higher *ApoE* ε4 allele frequency (6.25) % than those with severe disease (1.25) %. Major contribution of *ApoE* ε4 allele was with decreased susceptibility to LC/LF development cannot be ruled out; (OR) (CI95), 0.18(0.02 to 1.61)/0.19 (0.02 to 1.66) (p=0.12,0.13) for (ε3/ ε4) genotype and ε4 allelle frequency respectively. Although non statistically significant difference due to relatively small sample size. Our result support other studies for a possible genetic association between *ApoE* ε4 allele with a lower probability of progression to HCV-related liver cirrhosis.

Keywords: Apolipoprotein E, CHC, Cirrhosis, Genotype, HCV, Liver failure

Abbreviation: Apolipoprotein E (**ApoE**); Chronic hepatitis C virus (**CHC**); Hepatitis C Virus (**HCV**); Hepatocellular Carcinoma (**HCC**); Liver Cirrhosis (**LC**); Liver Failure (**LF**); Polymerase Chain Reaction (**PCR**); Restriction Fragment Length Polymorphism (**RFLP**); Single Nucleotide Polymorphism (**SNP**)

INTRODUCTION

Hepatitis C continues to be a major public health problem, during chronic hepatitis C, progressive fibrosis deposition occurs and this deposition ends in cirrhosis in 20-30% of Chronic Hepatitis C (CHC) carriers and 2.5%

of HCV-infected individuals develop Hepatocellular Carcinoma (HCC) later in life (Poynard et al., 1997; Bowen and Walker, 2005). The pathway of Hepatitis C Virus (HCV) assembly and secretion is closely linked to lipoprotein production and secretion, and HCV particles circulating in the blood associate with lipoproteins, thus, it is termed a Lipo-Viro Particle (LVP) (Nielsen et al., 2006; Hishiki et al., 2010; Aizawa et al., 2015). Moreover, HCV might gain entry into cells via a hitchhiker method with the lipoproteins (Agnello et al., 1999). Specifically, entry might involve LDL receptors (LDLRs) (Aizawa et al., 2015). The key lipoprotein molecules mediating these interactions are apolipoproteins (Sabile et al., 1999; Aizawa et al., 2015). Apolipoprotein E is a key molecule required for HCV entry and is one of the possible therapeutic targets for interrupting HCV infection. Apolipoproteins (Apo) are amphipathic protein on the surface of a lipoprotein particle, which help stabilize lipoprotein structure with numerous roles in regulating lipid and lipoproteins, apolipoproteins are classified into A,B, C, E (Aizawa et al., 2015). *ApoE* is polymorphic proteins, identifed in 1973 (Shore and Shore, 1973) and was first recognized for its role in cardio-vascular diseases (CVD) and widely searched not only for cardio-vascular disorders but also in relation to several other medical conditions such as neurodegenerative and autoimmune diseases. The key protein, playing many roles in lipid absorption, transport, local homeostasis in the vessel walls, and endothelial function (Sacre et al., 2003). *ApoE* plays a central role in cholesterol transport as reflected by the association of *ApoE* with a variety of lipoprotein size classes and the ability of *ApoE* to interact with two distinct hepatic receptors (LDL receptor and *A*poE receptor) (Ahn et al., 2012; Aizawa et al., 2015). "*ApoE* has additional roles, as a modulator of the immune function (Kelly et al., 1994) and also has direct effect on tissue macrophage recruitment, independently, of the lipoprotein metabolism. Several data on the role of *ApoE* in the regulation of inflammation were reported" (Aizawa et al., 2015).

ApoE is synthesized primarily by the liver and it is estimated that 20-40% of total *ApoE* is produced by extrahepatic tissues with the brain glial cells and macrophages expressing relatively high amounts, with lesser amounts produced by the kidneys, adrenals, spleen, testis and the skin (Eichner et al., 2002; Minihane et al., 2007). The human *ApoE* gene spans 3.7 kb including four exons and three introns (Das et al., 1985; Hixson and Vernier, 1990) and is located on chromosome 19 in a gene family that also contains the genes for apoC-I, C-I' (a pseudo- gene), and C-II (Hixson and Vernier, 1990; Myklebost and Rogne, 1988). *ApoE* exists in 3 major isoforms (ApoE2, ApoE3, ApoE4), determined by two single nucleotide polymorphisms (SNPs) in the *APOE* gene (rs7412 and rs429358, coded by three codominant alleles (ε 2, ε 3, ε 4). The three major alleles are responsible for three homozygous (ε2/ε2, ε3/ε3, 4/ε4), and three heterozygous (ε2/ε3, ε2/ε4, ε3/ε4) genotypes (Weisgraber et al., 1981; Zannis and Breslow, 1981). *Apoε3* seems to be the wild-type isoform with normal function. *ApoE ε2* and *ApoE ε*4 differ from *ApoE ε3* by a single amino acid substitution. While *ApoE ε3* contains cysteine at amino acid position 112 and arginine at position amino acid 158, the arginine is substituted by a cysteine in *ApoE* ε2 carriers and the cysteine is substituted by an arginine in *Apoε4* carriers. Additionally, two minor alleles of the gene, ε1 and ε5, exist but these are present in less than 0.1% of the population (Ordovas et al., 1987). Each isoform has specific functional properties including different susceptibilities to diseases (Myklebost and Rogne, 1988; Bennet et al., 2007; Villeneuve et al., 2014). The reasons for the diversity of outcomes of HCV infection are unclear. Different factors may affect the outcome of HCV (e.g. age, other coexisting virus infections, genetic predisposition etc).

In this study, we evaluate association of the different genotypes of *ApoE* and HCV induced liver cirhosis in sample size of chronic hepatitis Egyptian patients.

SUBJECTS AND METHODS

Ethical Statement

Informed written consent is obtained from all participants according to human ethics committee approval. The study protocol was reviewed and approved by the Ethics Committee of the Ain Shams University and the work done at Medical Research Center, Ain-Shams University Hospitals.

Subjects

One hundred and twenty subjects were included in our study and were categorized into three groups. The study was done in the period between June 2011 and February 2013. It included 80 Egyptian patients who were recruited from Internal Medicine and Hepatology outpatient clinics and inpatient at Ain Shams University Hospitals, in addition to a control group of 40 healthy volunteers.

Patient groups

Group I: Forty CHC with no signs of cirrhosis.
Group II: Forty CHC cirrhotic patients with signs of liver cell failure or portal hypertension according the modified Child-pugh score (C).

Control group

Forty age and sex matched healthy volunteers to deter-

Table 1. Patients characteristics of the study groups

	Control n=40 n(%)	CHC (non cirrhotic) n=40 n(%)	CHC (Cirrhotic/Liver Failure) n=40 n(%)	Chi-square	
				X^2	P-value
Sex					
Male	20(50)	24(60%)	21(52.5)	0.873	0.646
Female	20(50)	16(40)	19(47.5)		
Liver/US					
Cirrhosis	0	0	40 (100)	120	<0.001
HFL	0	0	0(0)		
No change	40 (100)	40 (100)	0		
Spleen/US					
Normal size	40 (100)	40 (100)	0	120	<0.001
Splenomegaly	0	0	40 (100)		
Encephalopathy					
No attack	40 (100)	40 (100%)	22 (55)	42.353	<0.001
Previous attack	0	0	18 (45)		
Ascites					
No/Ascites	40 (100)	40(100)	0	120	<0.001
Mild	0	0(100)	5 (12.50)		
Moderate	0	0	10 (25)		
Tense/Severe	0	0	25 (62.5)		
Child Pugh Score					
A			0	-	-
B			0		
C			40 (100)		

HFL Hepatic focal lesion Significance < 0.05

mine the prevalence of *ApoE* different genotypes in Egyptian population.

Exclusion criteria

Patients with liver cirrhosis due to other viruses or diseases other than Hepatitis C (e.g. Hepatitis B, Alcoholics….etc.) were excluded from the study.

Patients and controls were subjected to the followings:

- Full history taking and complete clinical examination.
- Laboratory investigations: Complete blood count, liver biochemical profile, viral markers.
- Radiological investigations for patients include: Abdominal ultrasonography, triphasic CT abdomen and/or dynamic MRI abdomen with MRI diffusion.
- The presence of the viral genome in serum was assessed by using the Amplicor reverse-transcription PCR assay (Roche, Welwyn, UK).
- The patients of the second group were subjected to liver biopsy to determine degree of fibrosis.
- *Apolipoprotein E* Genotyping for all the samples obtained from patients and control group was done by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method.

Methodology

ApoE genotyping was performed by extracting DNA from whole blood samples using DNA extraction kit supplied from Qiagen (Valencia, CA, and USA). Genotyping for *ApoE* polymorphisms was carried out as method described by (Hixson and Vernier, 1990). Amplification reactions were performed, DNA was amplified in a 50 µL reaction mixture using PCR master mix supplied by Qiagen. Thermo cycling was done using in a thermal cycler (Perkin-Elmer 9600, Cambridge, United Kingdom) according to the following cycle profile: initial denaturation for 2 minutes at 95°C, 40 cycles at denaturation 95°C (1 min.), annealing 60°C (1 min.), extention 72°C (2 min.) and for the final phase, there was an initial incubation at 72°C (5 min.). The primers were used for amplification by PCR, with the following sequences (Emi et al., 1988): Forward 5'- TAAGCTTGGCACGGCTGTCCAAGGA-3', Reverse 5'-ACAGAATTCGCCCCGGCCTGGTACAC-3'. Upon completion of PCR, the products were analyzed by electrophoresis on a 2% ethidium bromide-stained agarose gel. After PCR amplification of *ApoE* region, 5 units of HhaI (New England Biolabs). *HhaI* cleaves at GCGC encoding 112arg (ε4) and 158arg (ε3, ε4), but does not cut at GTGC encoding 112cys (ε2, ε3) and 158cys (ε2) (Table 1). *HhaI* was added directly to each reaction mixture for digestions of *ApoE* sequences for >3

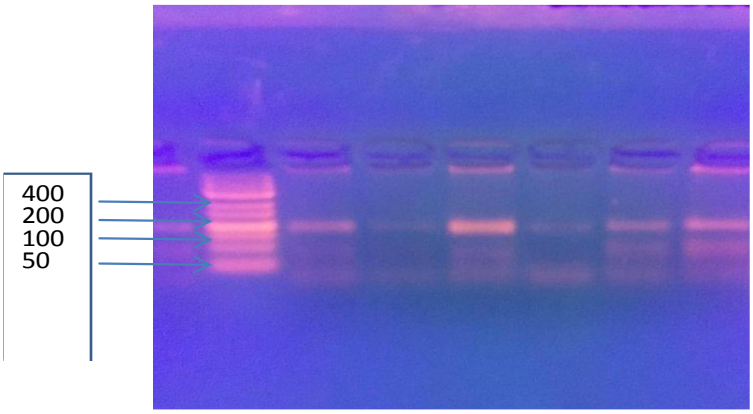

Figure 1. Electrophoretic separation of ***HhaI*** fragments after gene amplification of DNA from subjects with known *ApoE* isoforms. The ε3/ ε3 samples contained the 91 bp fragment (112cys), as well as 48 fragment, the 35 bp fragments (not shown). The fragment sizes (in bp) of a DNA standard (Thermo Scientific Gene Ruler Low Range DNA ladder) are shown to the left of the gel. The ε3/ ε4 samples not shown…..

hours at 37°C; blocking of the reaction was done at 65°C for 15 minutes. Each reaction mixture was loaded onto 8% Polyacrylamide Gel (1.5 mm thick and 25 cm long), and electrophoresed for 3 hours under constant current (45 mA). After electrophoresis, the gel was treated with ethidium bromide (0.2 mg/dl) for 10 minutes, and DNA fragments were visualized by UV illumination. The sizes of HhaI fragments were estimated by comparison with known size markers (Thermo Scientific GeneRuler Low Range DNA ladder).

Statistical analysis

The collected data was organized, tabulated and statistically analyzed using statistical package for social science (SPSS) computer package, version 17 (SPSS Inc, USA), running on IBM-compatible computer. Quantitative data were represented as mean, standard deviation (SD).

Qualitative (categorical) data were represented as relative frequency and percent distribution, Chi square (X^2) for comparison between groups was used. Allele frequencies are reported with their group percentages. The risk associated with the presence of the *ApoE*-4 allele was estimated for each diagnostic subgroup by using odds ratios (with their 95% confidence limits). For interpretation of results, p value ≤0.05 was considered statistically significant.

RESULTS

Baseline characteristics of CHC patients are demonstrated in (Table 1). Mean age was 45.85 ± 5.26in CHC (non cirrhotic) patients, 54.30 ± 10.38 in CHC (cirrhotics/LF) and 52.50 ± 10.57 in healthy controls without significant difference between groups (p>0.05). Non significant difference regarding sex between the studied groups. The study groups were also different regarding symptoms (hepatic encephalopathy and ascites), and imaging studies (ultrasonography for liver and spleen) with highly significant statistical difference (p<0.001).

The laboratory data among studied patients

On comparing the study groups as regards laboratory investigations, it was found that there was highly significant difference in TLC, Hb and Plt counts (p<0.001 for all parameters). As regards liver function tests, a significant difference in ALT, AST (p=0.03,0.04), and a highly significant difference in total bilirubin and serum albumin (p<0.001 for both parameters). As regards coagulation profile, there was highly significant difference between the study groups (p<0.001 for all parameters)**.**

Restriction enzymes HhaI digestion for genotype assays for *APOE* Gene

PCR amplification shows 244 bp fragments, each genotype was determind by fragments combination specific for each genotype after restriction enzymes *HhaI* digestion. ε3/ε3 sample contained the 91 bp fragment (112cys) and due arginine at amino acid position 158 inε 3, the 158 fragment was digested to 48 and 35 bp fragments in ε 3 allele, in addition, ε4/ε4 has arginine at position 112, so 91 bp fragment will digested to unique 72 bp and 19 bp (short to be detected). 38 bp, and (16bp and 18bp; short to be detected) (common fragment for all genotypes) (Figure 1).

Table 2. Distribution of the genotypes and alleles frequencies of *ApoE*in the studied groups

Genotypes	CHC (Non cirrhotic)		CHC (Cirrhotic/LF)		Controls		Chi-square	
	n	%	n	%	n	%	X^2	P-value
(ε2/2-3-4)/(ε 4/ ε 4)	0	0.00	0	0.00	0	0.00	2.83	0.24
(ε 3/ ε 3)	35	87.50	39	97.50	36	90.00		
(ε 3/ ε 4)	5	12.50	1	2.50	4	10.00		
Total	40	100.00	40	100.00	40	100.00		
Allele frequency	N	N	N	N	N			
ε 2	0	0.00	0	0.00	0	0.00	2.71	0.25
ε 3	75	93.75	79	98.75	76	95.00		
ε 4	5	6.25	1	1.25	4	5.00		
Total	80	100.00	80	100.00	80	100.00		

Note: n, number of subjects; N, number of alleles.

Table 3. The association between *ApoE* Gene Polymorphism and HCV progression to liver cirrhosis

	CHC	
	LC/LF vs Non cirrhotic	***P***
Genotypes	**OR (CI95%)**	
(ε2/2-3-4)/(ε4/ ε4)	-	-
(ε 3/ ε 3)	Reference	
(ε 3/ ε 4)	0.18 (0.02 to 1.61)	0.12
Allelle frequency	**OR (CI95%)**	
ε 2	-	-
ε 3	Reference	
ε 4	0.19 (0.02 to 1.66)	0.13

The genotypes and allelles frequency of APOEGene Polymorphism

Among the 120 subjects, the most common genotype was ε3/ ε3, accounting for (91.67)%, followed by ε3/ ε4 (8.33) %. The genotypes of ε2/2 2/3 2/4 and ε4/4 were not detected in our results. The study reported that; *ApoE* (ε 3/ ε 3) wild homozygous genotype (97.50)% (87.50)% (90) % in CHC with LC with signs of liver cell failure (LF), non cirrhotic patients and healthy control and *ApoE* heterozygous genotype (ε 3/ ε 4) (2.50)% (12.50)% (10.00) %in those groups respectively. The ε3 allele was the most common allele over represented in CHC LC/LF (98.75) % versus (93.75)% in non cirrhotic group. However, CHC non cirrhotic patients had a higher *ApoE* ε 4 allele frequency (6.25) % than those with severe disease (1.25) % (Table 2). Major contribution of *ApoE ε*4 allele was with deccreased susceptibility to LC/LF development can not be ruled out; (OR) (CI95), 0.18(0.02 to 1.61)/0.19 (0.02 to 1.66) (p=0.12,0.13) (ε3/ ε4) genotype and ε4 allelle frequency respectively (Table 3).

DISCUSSION

Hepatitis C virus infection is a major global health problem. HCV infection affects almost 3% of the world's population. More than 170 million people worldwide are infected with HCV (Shepard et al., 2005; Kamal and Nasser, 2008) with the highest prevalence in Egypt (15%) with genotype 4 being the most common (Kamal and Nasser, 2008; World Health Organization, 2007; El-Zanaty and Way, 2009). The consequences of chronic infection also vary (Wozniak et al., 2002); some individuals develop minor or no liver damage, whereas others suffer from progressive chronic hepatitis, leading to liver cirrhosis, and even HCC (Alter et al., 1992; Frank et al., 2000; Bostan and Mahmood, 2010; Wozniak et al., 2016) and high rates of infection are observed among persons in all age groups (Abdel-Aziz et al., 2000; Perz et al., 2006), the reasons for the diversity of outcomesof HCV infection are unclear, infection with a particular genotype is not thought to influence disease outcome. Alternatively, host factors may play a role; some of these factors include male sex, an older age at infection, increased alcohol intake, coinfection with HIV-1 or HBV (Simmonds et al., 1993; Zarski et al., 1998; Mohsen, 2001) and HLA types (Khakoo et al., 2004), genetic factors could be one of major influence.

ApoE is multifunctional protein with numerous roles in lipoprotein metabolism. The frequencies of the *apoE*

Table 4. Estimated worldwide distribution of human *ApoE* allele frequencies

Estimated worldwide human allele frequencies of ApoE based upon over 200 world populations and 50,000 people (highly variable depending upon population)			
Allele	ε2	ε3	ε4
Frequency	0 – 37.5%	8.5 – 98%	0 – 49%

alleles vary between different ethnicities. Several global studies on *ApoE* genotype frequency varied greatly among different population that *ApoE* (ε 3/ ε 3) genotype, the most common genotype in different population followed by (ε 4/ ε 4) and then (ε2/ ε 2) (Hallman et al., 1991). The ε3 allele is usually the most prevalent, present in 50-90% of individuals, whereas ε2 has the lowest frequency at 0-15%, and is even absent in some native populations. The ε4 allelic variant occurs at a frequency of 5-30% (Gerdes et al., 1996). *Also,* Farrer et al., (1997) stated that the estimated worldwide distribution of *ApoE ε4* allele was 13.7%. Another study by Eisenberg et al., (2010) Table 4.

Previous studies on *ApoE* genotypes revealed gene polymorphism and its role in susbtibility to different diseses and many infectious agnet; Hill et al., (2007) stated possible role of *ApoE ε2* in HCV viral clearance via defective binding of HCV lipoviral particle to cellular receptors involve in entery of this virus particle. In further contrast, *ApoE ε 2* is a risk factor for herpes simplex encephalitis (Wozniak et al., 2002). Whereas *ApoE ε 4* it is a risk factor for Alzheimer's disease in those harboring HSV1 in the brain, and a risk factor for cold sores in those harboring HSV1 in the peripheral nervous system (Itzhaki et al., 1997; Lin et al., 1998). Also, it confers a risk for reversible dementia and peripheral neuropathy in HIV-infected people (Corder et al., 1998).

ApoE ε4 is considered as an accepted risk factor for Alzheimer's disease and cardiovascular disorders. Recently, associations between *APOE ε4* and lower probability of progression to HCV-related liver cirrhosis has been demonstrated. The possible mechanism for *ApoE ε4* action, the amino acid arginine substitution results in an "interaction between *ApoE ε4* amino- and carboxyl- terminal domains, known as "domain interaction" (Mahley and Huang, 2009), this structural difference is believed to play a role in the altered function of Apoε4" (Villeneuve et al., 2014), another explanation stated by Davignon et al., (1988), *ApoE* ε4 allele is associated with increased serum levels of LDL, HCV entery through LDLRs, (Monazahian et al., 1999). The greater number of LDLR ligands in the serum of *ApoE* ε4 allelle carrier lead to competition for the receptor binding, therapy may prevent or reduce binding of the virus to hepatocytes and therefore reduce cell damage. In this study we investigated whether specific *ApoE* genotypes and HCV induced liver cirrhosis in Egyptian patients by PCR-RFLP. PCR amplification and Restriction isotyping methodology avoids the use of costly and time-consuming hybridization and sequencing techniques (Hixson and Vernier, 1990).

The study reported that ApoE heterozygous genotype (ε3/ε4) and ε4 allelle more frequent in CHC patients (non cirrhotic) than decompensated group with deccreased susceptibility to LC/LF development can not be excluded. Althogh non statistically significant difference due to relatively small sample size, with a relatively larger sample size, it may achieve statistical significance confirmed this figure. Compared to the study of Wozniak et al. (2002) in which *ApoE ε4* allele frequency was 6.5% in cirrhotic patients vs. 20% in non-cirrhotic patients,our results is similar to Wozniak's study in the concept that the cirrhotic group had a lower *ApoE ε4* allele frequency than the non-cirrhotic group, which further proves the hypothesis that *ApoE ε4* genotype may have a role in protection against HCV-induced liver cirrhosis. Another study by Mueller et al., (2016) reported lower-representation of *ApoE ε4* allele carriers in patients with CHC compared to the higher-representation of *APOE ε4* allele carriers in patients who spontaneously cleared HCV infection suggests a protective role of the *ApoE ε4* allele in the course of HCV infection.

The estimated worldwide distribution of *ApoE ε4* allele widely varied frequency, the prevalence of *ApoE ε4* allele frequency in our study 5.00%, this difference can be attributed to possible ethnic factors related to Egyptian population, or small sample.

CONCLUSION

The effect of *ApoE* on disease risk is complex. More studies still warranted to understand; the factors and mechanisms involved in *ApoE*-related interactions which could improve awareness of disease susceptibility, disease progression, disease prevention, and *APO E* status and therapeutic association. Assessing *ApoE* genotypes may give valuable information that help to guide medical decisions in clinical practice and for personalized medicine. Limitation of the current study, this study involving only 80 CHC Egyptian patients and 40 healthy controls, for this relatively small sample size and estimated worldwide overall prevalence *of ApoE ε4* allele frequency and the difference related to Egyptian population, probability and statistics may have been a limiting factor in the detection of the statistically significant association between *ApoE* gene polymorphism in CHC patiens. Our result support many different other

studies and highlight possible role of *ApoE* ε4 Allele for protection with lower probability of progression to HCV-related liver cirrhosis in Egyptian patients but further investigations with a planned prospective population based with different ethnicity with larger sample size are needed.

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Conflict of Intrest

The authors declare no conflict of intrest

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