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Impact of Apo E gene polymorphism on HCV therapy related outcome in a cohort of HCV Egyptian patients

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ABSTRACT

The functional apolipoprotein E (Apo E) gene polymorphism could be used as a determinant of outcome of HCV infection. This study aimed to demonstrate the impact of Apo E genotype on the response to HCV combined therapy. Material and methods: The study has been implemented on 125 individuals with persistent HCV infection and 120 cases with sustained virologic response (SVR). All participants were genotyped for ApoE gene polymorphism by a real-time quantitative PCR (qPCR). Results: Statistically significant differences were demonstrated regarding the Apo E genotypes between the two groups (P-value < .001) where the frequency of E3E3 was significantly higher among the chronic HCV-patients while E3E4 and E4E4 genotypes frequencies were higher among the SVR-subjects group and E3E3 genotype was associated with increased risk of chronicity (OR 4.7; 95% CI 1.9–12.1, P-value < .001). Moreover, There were statically significant differences regarding E3 and E4 alleles frequencies, where E3 allele display a higher frequency among the chronic HCV-patient group while the SVR-subjects group showed higher frequency of E4 allele and the carriers of E3 allele have 1.4 times more risk to develop chronicity than those with E4 allele (OR 1.4; 95% CI 1.0–2.0, P-value < .05). Meanwhile the protective E2 allele was absent in all infected participants. Conclusion: This study supports the hypothesis of the protective impact of Apo E4 allele that favors viral clearance of HCV infection and its recovery after combined therapy, while the Apo E3 allele is considered as a particular risk factor for the chronicity in HCV patients and resistance to therapy. Whereas the Apo E2 allele confers a resistance to HCV infection at a time of exposure.

Contents

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1. Introduction

A unique feature of HCV is that both cholesterol metabolism and fatty acid biosynthetic pathways are among the fundamental stones for both RNA replication and virion assembly in host cells [1]. Apolipoprotein E (Apo E), a ligand for low density lipoprotein receptor (LDLr), is now prescribed as an irreplaceable component of the HCV–host lipid interaction through involvement at several stages in the viral lifecycle, including intracellular viral assembly [2,3]. Apo E has been well described as a main regulator of many steps of lipid (cholesterol and triglyceride) and lipoprotein metabolism, including synthesis and secretion of very low density lipoproteins (VLDL), VLDL hydrolysis to produce low density lipoproteins (LDL) and receptor mediated removal of triglyceride-rich lipoprotein remnants (VLDL and Chylomicrons remnants) by the liver. Lipoviral Particles (LVP) had been identified as low-density HCV virions packaged as LVPs with densities similar to that of the very-low-density lipoprotein (VLDL) and abundance of Apo E on their surface detected by electron microscopy [4], shedding the light on the implication of the Apo E in mediating HCV infectivity via lipoprotein receptors [5]. Furthermore, Apo E have been detected in the low-density fractions of the HCV RNA-containing particles which had been declared as the fractions carries the probability of being infectious [6]. Endocytosis of HCV is mediated by low density lipoprotein receptor (LDLr) and Scavenger receptor B1 (Sc-B1). LDL r normally transports 2 different classes of cholesterol containing lipoprotein particles (LDL & VLDL) which contain multiple copies of Apo E [7]. While Sc-B1 which is expressed primarily in the liver recognizes a broad variety of lipoprotein ligands (HDL, LDL, VLDL and oxidized LDL) [8].

Apo E is defined as a polymorphic protein arising from three alleles. The human Apo E gene was widely studied and described to be located on chromosome 19, closely linked to the Apo C-I/C-II gene complex [9]. The three major alleles, termed Epsilon-2, Epsilon-3 and Epsilon-4 have been reported [10]. The apoE2, apoE3 and apoE4 protein isoforms, corresponding products of these alleles, differ only by a single amino acid at two residues. Where Apo E2 contains cysteine at two residue 112 and 158 and Apo E4 has arginine at both positions while apoE3 has cysteine at residue 112 and arginine at residue 158 [11]. Less than 0.1% of the population have additionally, two minor alleles of the gene, ε1 and ε5. Three homozygous (E2/E2, E3/E3, E4/E4) and three heterozygous (E2/E3, E2/E4, E3/E4) genotypes will be determined by these three major alleles [12].

This study was conducted to determine the relevance of the Apo E gene polymorphism on the outcome of HCV infection after combined therapy and whether these allelic variants could be used as risk biomarkers of HCV infection prognosis.

2. Material and methods

2.1. Patients

Patients with HCV-genotype 4 infection attended for a morning fasting blood sample. Individuals that were alcohol dependent, on concurrent lipid lowering or co-infected with hepatitis B, schistosoma, or human-immunodeficiency virus, were excluded from the start. A total of 245 patients were included in this study, 125 chronic HCV cases (non responders to Interferon and ribavirin therapy), 120 patients who achieved a SVR (those had a negative HCV-RNA after 6 months of completing 48 weeks therapy) [13].

The participants were recruited from hepatology clinic of National Research Centre and Liver Institute of Cairo University. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the National Research Centre (Egypt) ethical committee and patients provided written informed consents to participate.

2.2. Laboratory investigations

A peripheral blood sample was obtained from all participants. An immediate centrifugation was done for 10 min at 5000 rpm at 4 °C. The centrifuged serum was transferred into sterile tubes. All samples were stored at −20 °C until assay. One ml of venous blood sample was collected in EDTA vials for the extraction of genomic DNA.

Total cholesterol, HDL-cholesterol (HDL-C), triglycerides and very low density cholesterol, were measured by standard automated enzymatic methods using an Olympus AU400 analyser (Olympus, Diagnostica, Japan). LDL cholesterol (LDL-C) was calculated using the Friedewald equation [14]. The serum LDL-c was calculated by this formula [15] as TG level did not exceed 400 mg/dl: LDL-c = total cholesterol − (TG/5 + HDL-c).

2.3. Hepatitis C virus detection and genotyping

The presence of HCV antibodies in serum was detected by third-generation enzyme linked immunosorbent assay ELISA; CTK-Bioteck-USA).

Viral RNA was extracted from patient's plasma using the QIAamp Viral RNA Kit (Qiagen Hilden, Germany) according to the manufacturer's protocol. HCV RNA was determined by Toyobo RNA-direct real time PCR kit on SLAN Real Time PCR Detection System, LG Lifescience, Korea.

The HCV genotype was defined by the reverse line probe assay (INNO-LIPA v.1.0, innogenetics, Ghent, Belgium) according to the manufacturer's instructions. This is the most widely used methods for HCV genotyping where the 5′ UTR of HCV is amplified with biotinylated primers, after which the PCR product is hybridized to a membrane impregnated with genotype-specific probes and detected with streptavidin linked to a colorimetric detector [16]. The LiPA-I kit included 17 probes: generic 1 (2 probes), 1a, 1b, generic 2 (2 probes), 2a (2 probes), 2b (2 probes), 3a (4 probes), and 4/5 (3 probes) [17].

2.4. Apo E genotyping

A real-time qPCR strategy with SYBR** Green I was used that rapidly genotype human Apo E haplotype alleles according to the methodology described by Andre et al., 2004 [18] with slight modifications. Apo E haplotype determination based on the differential amplification of alleles using designed primer sets that contain specific terminal bases for SNP interrogation. Amplification is performed on Applied Biosystems 7500 Instrument.

Primers used in the reaction: the 3′ terminal base of each primer corresponds to a specific SNP allele

Forward primers correspond to the SNP at position 3937:

F(T):5′GGA-CAT-GGA-GGA-CGT-G(T)-3′
F(C):5′GGA-CAT-GGA-GGA-CGT-G(C)-3′

Reverse primers correspond to the SNP at position 4075:

R(G):5′-GGT-ACA-CTG-CAG-GC(G)-3′
R(A):5′-GGT-ACA-CTG-CAG-GC(A)-3′

The primers amplified a 169 bp amplicon from total genomic DNA.

Reactions using primers that lack the SNP-interrogating nucleotide were performed as positive controls

F(pos):5′-GGG-ACA-TGG-AGG-ACG-TG-3′
R(pos):5′-TGG-TAC-ACT-GCA-GGC-3′

An amount of 2.0 ng/ul DNA was mixed with 10 Pmol of for ward and reverse primers (1 μL from each one) and 10 μL of Quantitect SG PCR mix. PCRs were carried out in a total volume of 20 μL/well, using StepOne™ Real-Time PCR System (Applied Biosystems, CA). The PCR amplifications were subjected to the following cycling parameters: 95 °C for 15 min, followed by 50 cycles of 94 °C for 15 s for denaturation and 60 °C for 30 s for annealing and 72 °C for 30 s for extension. Finally, 72 °C for 10 min.

2.5. Haplotype determination

Each DNA sample was run in five PCR reactions: four reactions with different combinations of SNP-interrogating forward and reverse primers and a positive control reaction. Monitoring product accumulation during the exponential phase of amplification, in contrast to traditional end-point analysis, is important for this protocol, since reactions with primer matches or mismatches may eventually accumulate equivalent amounts of products. The C(t) value; an indication of the cycle number at which a sample's fluorescence signal exceeds background red for each DNA sample to determine haplotype. With lower C(t) values, SNPs of haplotype match the 3-ultimate nucleotides of both forward and reverse primers. Such values will always be obtained in reactions containing the positive control primers [F(pos)-R(pos)]. While higher C(t) values, SNPs of haplotype don't match primers. Such values will always be obtained in reactions containing the negative control primers (F(C)-R(A).

2.6. Statistical analysis

Statistical analysis: Data were analyzed using SPSS© Statistics version 17 (SPSS Inc., Chicago, IL). Normality of numerical data distribution was examined using the D'Agostino-Pearson test. Parametric numerical variables were presented as mean ± SD and intergroup differences were compared using the unpaired t test in comparing 2 groups and one-way analysis of variance (ANOVA) followed by Post Hoc test for the comparison of more than 2 groups. The distributions of the allelic and genotypic frequencies of the studied SNP respected the Hardy–Weinberg equilibrium. Categorical variables were presented as number and percentage and intergroup differences were compared using fisher's exact test. Categorical variables were compared using the chi squared test and odds ratio (OR) with a 95% confidence interval (95% CI) for trend. P-value < 0.05 was considered statistically significant.

3. Results

HCV patients genotype 4 were recruited to this study. One hundred and twenty five were proved to be chronically infected, while 120 of the participants were SVR with age ranged between 20 and 50 years.

Table 1
Comparison of lipid profile between the chronic cases and subjects with SVR.

	Chronic HCV (n = 125)	SVR (n = 120)	P-value
T.Chol. (mg/dl)	157.6 ± 41.79	181 ± 45.32	P < .001
Trig. (mg/dl)	113.2 ± 55.66	126.6 ± 62.56	NS
HDL (mg/dl)	40.4 ± 13.89	43.1 ± 13.62	NS
LDL (mg/dl)	95.3 ± 37.03	112.3 ± 41.3	P < .001
VLDL (mg/dl)	23.3 ± 11.38	24.4 ± 11.38	NS

Values are presented as mean ± SD. P-value < .05 is statistically significant. NS: Non significant.

Comparison of lipid profile between the chronic HCV cases and subjects with SVR were summarized in Table 1. There were statistically significant differences between the two groups regarding cholesterol and LDL serum levels where patients with chronic HCV infection had lower level of cholesterol and LDL compared to the subjects with SVR (P < .001), while no statistically significant differences as regards triglyceride, HDL and VLDL serum levels were demonstrated.

The distribution of Apo E genotype frequencies, allele frequencies and risk association were compared between the two groups and summarized in Table 2. Statistically significant differences were demonstrated regarding the Apo E genotypes between the two groups (P-value < .001) where the frequency of E3E3 was significantly higher among the chronic HCV-patients while E3E4 and E4E4 genotypes frequencies were higher among the SVR-subjects group (P-value < .001) and E3E3 genotype was associated with increased risk of chronicity (OR 4.7; 95% CI 1.9–12.1). Moreover, There were statically significant differences regarding E3 and E4 alleles frequencies, where E3 allele display a higher frequency among the chronic HCV-patient group while the SVR-subjects group showed higher frequency of E4 allele (P-value < .05). Demonstration of the risk association of the different Apo E alleles and HCV chronicity among the studied groups showed that carriers of E3 allele have 1.4 times more risk to develop chronicity than those with E4 allele (OR 1.4; 95% CI 1.0–2.0). Meanwhile the protective E2 allele was absent in all infected participants.

Comparison of the serum levels of lipid profile among the different genotypes were demonstrated and shown in Table 3. Although the serum level of total cholesterol and LDL is lower in E3 carrier allele than those with E4 carriers, yet the difference is not statistically significant.

Following ANOVA test, Post Hoc test was done to differentiate between individual genotypes concerning serum levels of lipid profile, there was no statistically significant difference between individual genotypes (P value > .05).

4. Discussion

Apolipoprotein E – a major structural LDL component and natural ligand of LDLR – which is intimately linked to plasma lipoprotein metabolism and may have an impact on the course of HCV infection. Three isoforms of Apo E have been identified E2, E3 and E4. Whereas the salt bridge formation within the proteins could be affected by the amino acid substitutions, which modulate the lipoprotein preference, protein stability and isoform receptor binding activities, the apoE genotypes may have a significant impact on HCV pathology and prognosis [19].

Linkage of the functional host-specific genetic polymorphisms within Apo E may influence the interaction between the host and HCV, and subsequently the outcome of HCV infection [20].

The present study investigated the possible link between Apo E genetic polymorphisms and the outcome of HCV infection and demonstrated statistically significant differences concerning the

Table 2
The frequency distribution and risk association of Apo E genotypes and alleles among the studied groups.

Gene	Chronic HCV-patients (n = 125)	SVR-subjects (n = 120)	[a]OR (95% CI)	P-value
Genotypes				
E3E3	25 (20%)	6 (5%)		
E3E4	99 (79%)	111 (92.5%)		<0.001
E4E4	1 (0.8%)	3 (2.5%)		
E3E3 (n = 31)	25 (20%)	6 (5%)		
			4.7 (1.9–12.1)	<0.001
E3E4 + E4E4				
(n = 214)	100 (80%)	114 (95%)		
E4E4	1 (0.8%)	3 (2.5%)	1 (Reference)	1 (Reference)
E3E4	99 (79%)	111 (92.5%)	0.37 (0.04–3.65)	0.39
E3E3	25 (20%)	6 (5%)	0.08 (0.07–0.9)	0.04
Alleles				
E3 (n = 280)	154 (61.6%)	126 (52.5%)		
			1.4 (1.0–2.0)	<0.05
E4 (n = 210)	96 (38.4%)	114 (47.5%)		

Data were evaluated by the gene counting method. Values are presented as percentage. P < .05 was statistically significant.
[a] Odd's ratio was used.

Table 3
Comparison of serum levels of lipid profile among the different Apo E genotypes.

	E3E3 (n = 31)	E3E4 (n = 210)	E4E4 (n = 6)	P-value
T.Chol. (mg/dl)	156.7 ± 39.2	170.6 ± 45.2	178.8 ± 22.1	0.3
Trig. (mg/dl)	111.2 ± 54.7	119.5 ± 56.5	110.5 ± 47.9	0.7
HDL (mg/dl)	38.4 ± 13.3	41.9 ± 13.2	38.0 ± 15.3	0.3
LDL (mg/dl)	95.2 ± 36.3	104 ± 39.4	113.8 ± 24	0.4
VLDL (mg/dl)	22.3 ± 10.9	24.0 ± 11.5	22.0 ± 9.6	0.6

Data are presented as mean ± SD. P is significant if it is <.05.

distribution of Apo E genotypes between the HCV-patients and SVR-subjects (P-value < .001) where the frequency of E3E3 was significantly higher among the chronic HCV-patients while E3E4 and E4E4 genotypes frequencies were higher among the SVR-subjects group (P-value < .001) also, E3E3 genotype was associated with increased risk of chronicity (OR 4.7; 95% CI 1.9–12.1). Moreover, There were statically significant differences regarding E3 and E4 alleles frequencies, where E3 allele display a higher frequency among the chronic HCV-patient group while the SVR-subjects group showed higher frequency of E4 allele (P-value < .05), moreover the carriers of E3 allele have 1.4 times more risk to develop chronicity than those with E4 allele (OR 1.4; 95% CI 1.0–2.0). Meanwhile the protective E2 allele was absent in all infected participants.

These findings not only assuming that that Apo E3 allele was associated with chronic outcome of HCV infection but also conferred a better chance of Apo E4 allele carriers to recover after convenient therapy and highlights the already established hypothesis that decreased LDL-R expression in E4 vs. E3 carriers could diminish viral maturation [21].

In concordance with this result, Price et al., 2006 who reported that E3E3 genotype is associated with highest risk of developing a chronic HCV infection in Caucasian hepatitis C patients, while E4 alleles is favoring viral clearance (the odds ratio was 0.59) [22]. Likewise, Mueller et al., 2016 concluded reduction of the susceptibility to chronic HCV infection in Apo E4 allele carriers [20]. Moreover, Teama et al., 2016 supported the possible genetic association between ApoE 4 allele with a lower probability of progression to HCV-related liver cirrhosis [23]. With the same respect, Kuhlmann et al., 2010 reported that the HCV-specific protective effect of apoE4 genotype and better outcome of chronic HCV infection justified by the slower fibrosis progression among E4 carriers [24].

Furthermore, the absolute lack of E2 allele expression in both chronic and SVR cases, supports the protective role of the E2 allele against HCV infection at a time of exposure. However this hypothesis could not be entirely proved by this study due to lack of healthy individuals participation. Similarly, Price et al., 2006 found a lack of E2/E2 genotype among patients who are HCV antibody positive, assuming that the E2/E2 genotype may grant a relative resistance for stabilizing HCV infection in exposed individuals [22]. Sheridan et al., 2009, also hypothesize that the E2 allele may protect against viral infection by means of defective engagement of HCV to the cellular receptors such as LDL receptors [25].

In contrast to these findings, no differences in apoE allele frequencies between the HCV RNA-positive group and the HCV RNA-negative individuals while studying the impact of apoE genotype on the outcome of HCV infection among both HCV patients with chronic infection or cleared infection by Wozniak et al., 2002 [26]. Nevertheless, the same study revealed significantly lower E4 allele frequencies in patients with severe versus mild inflammation when they examined the apoE genotype impact on the severity of HCV-induced liver damage [26].

Interactions between chronic hepatitis C virus infection and lipid metabolism have been described in some studies. A higher prevalence of hypocholesterolemia and low LDL levels in HCV-infected patients was reported in many studies [27,28].

This study revealed that patients with chronic HCV had significantly lower total cholesterol and LDL serum levels compared to the group with SVR (P-value < .001, for both). The intrahepatic cholesterol synthesis might be impaired due to HCV replication through two possible pathways; first, decreasing the availability of a substantial intermediate (geranylpyrophosphate) for synthesis of cholesterol, Second, cholesterol could be utilized for intracellular membranes synthesis during viral replication. Both mechanisms results in a negative feedback on the amount of cholesterol available for physiologic intracellular processes and for peripheral delivery via VLDL, consequently serum cholesterol levels will be decreased. Also, the decrease of serum LDL levels in HCV infection might occur due to reduction of available intracellular cholesterol that may also increase LDL receptors resulting in increased

intrahepatic LDL with subsequent reduction of serum LDL level [29]. Although no statistically significant difference could be elucidated between the Apo E genotypes concerning cholesterol and LDL serum levels, yet their levels was lower in E3 allele carriers the which confreres chronicity. Similar results were also obtained by Marzouk et al., 2007, Corey et al., 2009 and Nashaat et al., 2010 [29–31]. Also, no statistically significant differences between the two groups as regards HDL, VLDL and triglyceride levels could be demonstrated which agree with Corey et al., 2009 [30], but disagree with Marzouk et al., 2007 & Perlemuter et al., 2002 who found a drop in these levels among chronic HCV infected patients [29,32].

These results shed new light on the exploitation of the functional major role of ApoE in the production of LVP, presumably by direct interaction with viral envelope glycoproteins. Lee et al., 2014 suggested that ApoE acts at a belated step of assembly, such as particle maturation and infectivity, where ApoE depletion affected neither the formation of nucleocapsids nor their envelopment [33]. Further characterization of the LVP have shown that HCV-LVP binding, hepatocyte cell lines entry and upregulation of the LDL receptors with consequent increases their internalization could be blocked by anti-apolipoprotein E [34].

Both biologically conceivable and statistically significant demonstrated in this study and if confirmed in a corroboration study, the interaction of ApoE with HCV could be considered reliable in the development of novel therapeutic strategies.

Conclusion: This study supports the hypothesis of the protective impact of Apo E4 allele that favors viral clearance of HCV infection and its recovery after combined therapy, while the Apo E3 allele is considered as a particular risk marker for the chronicity in HCV patients and resistance to combined therapy. Whereas the Apo E2 allele confers a resistance to HCV infection at a time of exposure.

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Conflict of interest

None declared.

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