RESEARCH ARTICLE

Role of ApoB-516C/T promoter gene polymorphism in the risk of Hepatitis C virus infection in Egyptian patients and in gender susceptibility

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At least 1 in 10 of the Egyptian population aged 15-59 is burdened with hepatitis C virus (HCV) infection, stamping Egypt the highest country harboring HCV worldwide. Considerable evidence supported the involvement of host genetic factors in the pathogenesis of HCV and the possibility of implementation in target therapies. *ApoB* gene polymorphisms are postulated to affect the susceptibility of HCV infection. Hence, we aimed to evaluate the relationship between ApoB-516C/T promoter gene polymorphism and HCV infection in a cohort of Egyptian patients and to explore whether higher levels of low-density lipoprotein (LDL) might compete with lipoviral particles (LVP) in the binding to LDL receptor (LDLR), thus escaping infection. Ninety-seven HCV patients and 96 matched controls were enrolled in this study. We genotyped ApoB-516C/T using PCR-RFLP method. ApoB concentrations were measured by immunoturbidimetric assay. The genotype and the allele frequencies of ApoB-516C/T promoter gene polymorphism in cases were statistically insignificant compared with healthy individuals ($P = 0.109, 0.125$, respectively). Sex stratification showed significantly lower counts of C/T genotype in female patients compared with female controls ($P = 0.011$, OR = 0.132, 95% CI = 0.026-0.657). Significantly higher levels of LDL and ApoB were detected in the control group ($P < 0.001$). This study shows that the ApoB-516C/T promoter gene polymorphism has no impact on the risk of HCV infection. However, the C/T genotype may be a protective factor for our female cohort. Further studies with larger samples are needed to verify this genetic gender diversity. Additionally, high levels of LDL and ApoB might prevent HCV infection.

KEYWORDS
ApoB promoter gene polymorphism, Egypt, HCV, LDLR, LVP

1 | INTRODUCTION

Hepatitis C virus (HCV) infection is the most frequent cause of chronic liver diseases including cirrhosis, liver failure, and hepatocellular carcinoma accounting for substantial morbidities and mortalities. Egypt has the largest load of HCV infection in the world. About 14.7% of Egyptian populations are infected with HCV.[1] As quoted by the

WHO representative in the country "Just about every family in Egypt is touched by hepatitis C" and reports that this highly infectious blood borne virus kills an estimated 40 000 Egyptians every year.[2] That is to say, HCV infection in Egypt has reached "The perfect storm."

A unique aspect of HCV particles, derived from infected subjects, is their tight association with low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL); collectively known as lipoviral particles (LVP). As lipoprotein-like particles, they are assumed to be surrounded by a phospholipid monolayer enclosing the core enriched in triglycerides and cholesterol esters. They are recognized by host

antibodies and are associated to varying extents with protein components of lipoproteins; ApoB-48, ApoB-100, ApoC-1, and ApoE.[3,4] Lipids and/or lipoprotein-related lipids for active infection by HCV may be linked with facilitated virus binding, entry, and/or fusion.[5]

Many efforts had been made in the identification of receptors involved in HCV entry into hepatocytes. Several cell surface molecules have been proposed as putative receptors for virus entry, such as CD81 tetraspanin, scavenger receptor class B type I (SR-BI) and LDL receptor (LDLR).[6] The LDLR is a mosaic protein of 839 amino acids that mediates the endocytosis of cholesterol-rich LDL. It is a cell-surface receptor that recognizes the lipoprotein; ApoB-100.[7]

ApoB is a structural protein moiety of plasma LDL which has an important role in the adjustment of the levels of plasma LDL.[8] The genetic polymorphisms of *ApoB* have been investigated intensively and most researches focus on the involvement of *ApoB* polymorphism in familial hypercholesterolemia,[9] atherosclerosis,[10] and coronary artery disease.[11] Being a supposed ligand for the HCV presumed receptor LDLR, the *ApoB* polymorphisms have become an attractive candidate for the study of their association with the HCV infection. Within this context, switch of C to T on the promoter region at −516 of *ApoB* gene has been recognized and this functional polymorphism was established to produce a major increase in the circulating levels of LDL and ApoB.[12–14]

From another aspect, gender variations in the incidence of viral hepatitis have been reported with lower incidence recorded among females. In 2008, a Demographic Health Survey (DHS) carried out in Egypt for HCV antibody testing, showed that HCV antibody prevalence was higher in males compared to females.[15] This difference has stimulated an interest in the implementation of gender-specific medicine, where the genetic diversity between different genders is taken into consideration.[16]

This study aims to investigate the association between the ApoB-516C/T promoter gene polymorphism and the risk of HCV in a cohort of Egyptian patients, and to estimate the genotypes distribution and risk conferred in both sexes. Also, to explore whether higher levels of LDL and ApoB might compete with LVP in the binding to LDLR, thus avoid HCV infection.

2 | SUBJECTS AND METHODS

2.1 | Subjects

Ninety-seven patients with HCV infection were enrolled in the study. All patients were positive for HCV antibody using a second-generation enzyme-linked immunosorbent assay and tested positive for HCV RNA with polymerase chain reaction (PCR). Ninety-six age, sex, and ethnically matched healthy individuals tested as negative for HCV antibody were also included as controls. Both patients and controls were not exposed to previous surgeries or blood transfusions or tattooing but underwent frequent dental procedures, and shared shaving razors, nail files, hair trimmers, and scissors at barber shops. Exclusion criteria were patients tested positive for hepatitis B virus (HBV), hepatitis D virus (HDV), human immunodeficiency virus (HIV),

and other pathogens of liver disease, also those with a history of metabolic or endocrine diseases affecting lipid profile are excluded.

Informed consents were obtained from subjects and the ethnicity of these subjects is known as Egyptians. The patients were recruited from the Tropical Medicine Department at Kasr Al-Ainy Hospital; Cairo University. The study was approved by the hospital's ethical committee (according to the WMA Declaration of Helsinki).

All patients and controls were subjected to the following: 1) Proper history taking including age, sex, residence, history of diabetes mellitus (DM) or hypertension (HTN); 2) full physical examination including full abdominal examination focusing on the palpation of liver and spleen as well as percussion of ascites; 3) laboratory investigations including, total cholesterol (TC), triglycerides (TG), high-density lipoprotein (HDL), low-density lipoprotein (LDL) and ApoB concentrations; and 4) *ApoB* genomic DNA extraction and analysis of ApoB-516C/T promoter gene using PCR-RFLP.

2.2 | Methods

2.2.1 | Sampling

Six milliliters of venous blood were collected under complete aseptic precautions from each subject after an overnight fasting of at least 12 h. Three milliter of the collected blood were dispensed into a labeled vacutainer containing ethylene diamine tetra-acetic acid (EDTA) for DNA extraction and analysis of ApoB-516C/T promoter gene using PCR-RFLP. Samples were immediately stored at −20°C till the time of DNA extraction and analysis. The other 3 ml of blood were collected in a labeled dry vacutainer, left for 10 min to clot and then centrifuged at 3000 rpm for 5 min. The separated serum was divided into aliquots for measurement of ApoB, total cholesterol (TC), triglycerides (TG), HDL, and LDL. Hemolyzed samples were discarded, repeated freezing, and thawing was avoided.

Serum lipids were measured by colorimetric end point using spectrophotometer 5010 at wavelength 546 for TC, TG and HDL, then LDL was calculated according to this equation {(TG/5 + HDL)−T.Ch}.[17] ApoB concentrations were measured using Cobas Integra 800 by an immunoturbidimetric assay.

2.3 | Genotyping

Genomic DNA was extracted from EDTA anticoagulated whole blood within 3-4 weeks after sampling according to manufacturer protocol using Gene JET Whole Blood Genomic DNA Purification Mini Kit (50 preparation) (4×) (catalog number: K0781-Thermo Scientific, Waltham, MA) which utilizes silica-based membrane technology in the form of a convenient spin column, eliminating the need for expensive resins, toxic phenol-chloroform extractions, or time-consuming alcohol precipitation. The standard procedure takes less than 20 min following cell lysis and yields purified DNA greater than 30 kb in size.

Genotyping of the ApoB-516C/T promoter gene was carried out by using a polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) assay 21. For quality control, genotyping was repeated for random samples in each group to confirm our results.

PCR assay was carried out for DNA amplification using the following primers: Forward primer: 5′GCTGGGGTTTCTTGAA-GACA3′, reverse primer: 5′CAAGCGTCTTCAGTGCTCTG3′, spanning the *ApoB* promoter region containing −516C/T site. All the primers were provided by Invitrogen (Waltham, MA).

PCR reactions were carried out according to manufacturer protocol using ready to use master mix (Dream Taq Green PCR Master Mix 4X) (catalog number: K1081-Fermentas, Waltham, MA) comprising 4 mM MgCl2, 0.05 U/μL of Dream Taq Gold Polymerase, Dream Taq Green buffer containing a density reagent and two dyes to monitor electrophoresis progress with 0.25 μM of each primer, in a 25-μL reaction. The tubes were then placed in the thermal cycler (Perkin Elmer 9600, Singapore). The PCR cycle program was as follows: 15 min at 94°C, followed by 35 cycles of 30 s at 96°C for denaturation, 30 s at 63°C for annealing, 30 s at 72°C for extension, and at last 7 min at 72°C for final extension.

PCR products (10 μL) were loaded on agarose gel (2%) and electrophoresis was carried out. The gel was visualized under ultraviolet light after staining with ethidium bromide. DNA was run together with 50 base pairs ladder (Supplied by Bio Basic Inc*Pack size: 50 μg [0.5 μg/μL], Markham, Ontario, Canada).

Ten microliters of the amplified PCR products were then digested using 1ul of the restriction enzyme FastDigest *Ear1* restriction endonuclease (catalog number: FD0234-Thermo Scientific, Waltham, MA) together with 17 μL nuclease-free water and 2 μL 10× fast digest buffer according to the manufacturer protocol. The mixture was mixed gently and spun down. It was incubated at 37°C for 15 min. The digestion products were resolved on a 2% agarose gel containing ethidium bromide and visualized under ultraviolet light to detect the amplified segment.

The restriction enzyme *EarI* does not cut the homozygous C/C allele, so the 422 bp amplicon remains unaltered after incubation with the enzyme. Whereas for the homozygous T/T allele, it contains a recognition site for the *EarI* restriction enzyme, so the digestion yields two DNA fragments 306 and 116 bp in length. In the case of the heterozygous C/T allele, incubation of the PCR amplification product with *EarI* yields three DNA fragments 422, 306, and 116 bp in length.

2.4 | Statistical Methodology

Data were statistically described in terms of mean ± standard deviation (±SD), frequencies (number of cases) and percentages when appropriate. Genotyping results were estimated by direct counting. Genotype counts and allele frequencies were compared between patients and controls by Chi-square (χ^2) test. The Hardy-Weinberg equilibrium was assessed among controls using the χ^2 test. The Comparison of numerical variables between the study groups was done using Student *t*-test for independent samples in comparing 2 groups when normally distributed and Mann-Whitney *U*-test for independent samples when not normally distributed. Risk estimation was documented in terms of Odds ratio (OR) with 95% confidence interval (CI). A *P*-value <0.05 was considered statistically significant. All statistical calculations were done using computer programs SPSS

(Statistical Package for the Social Science; SPSS Inc., Chicago, IL) version CCFXn 15 for Microsoft Windows.

3 | RESULTS

As regard the demographic data, there was no significant difference between both HCV-infected patients and healthy individual groups. On the other hand, all serum lipid data and ApoB levels showed highly significant differences between both groups (Table1).

The comparison between the HCV patients and the healthy controls as regards ApoB −516 genotypes and alleles showed no statistically significant difference between both groups (Table 2). Genotype distribution of *ApoB* polymorphism in controls was in accordance with Hardy-Weinberg equilibrium ($P > 0.05$). When stratified by gender, the ApoB −516C/T genotype was associated with a decreased risk for the development of HCV infection compared with the C/C genotype in the female group (Table 3).

The 2 groups (The C/C and C/T genotypes of ApoB) of HCV patients were compared regarding TC, TG, HDL, LDL, and ApoB. However, we did not find any statistically significant differences between the two groups as regards all these points of comparison (Table 4).

4 | DISCUSSION

Therapeutic inhibition of HCV entry is an attractive target for future antiviral treatments. Indeed, the administration of monoclonal antibodies targeting important HCV entry factors has been established to limit HCV infection in human chimeric mice.[4] The ApoB molecule is considered a common dominator for LDL and LVP which accounts for subsequent competition for binding to the LDLR. On that account, attention has been paid to the role of the *ApoB* polymorphisms in the susceptibility to HCV infection.[18]

TABLE 1 Demographic and serum lipid data in HCV infected patients and healthy individuals

	Case (*n* = 97)	Control (*n* = 96)	*P*-value
Sex			
Male	66 (68.0%)	64 (66.7%)	0.879
Female	31 (32.0%)	32 (33.3%)	
Age	46.2 ± 5.0	45.8 ± 4.4	0.510
TC (mg/dL)	150.5 ± 22.1	211.3 ± 35.1	<0.001
TG (mg/dL)	140.8 ± 28.2	155.9 ± 19.8	<0.001
HDL (mg/dL)	35.0 ± 6.1	42.1 ± 4.3	<0.001
LDL (mg/dL)	79.4 ± 23.0	137.2 ± 14.7	<0.001
ApoB (mg/dL)	67.9 ± 14.9	89.6 ± 9.8	<0.001

TC, total cholesterol; TG, triglycerides; HDL, high-density lipoprotein; LDL, low-density lipoprotein; ApoB, Apolipoprotein B.
The data are listed as the mean ± SD or numbers of patients when appropriate.

TABLE 2 Genotypic counts and allelic frequencies of the ApoB −516 polymorphism between cases and controls and their associations with the risk of HCV

	Cases (n = 97) N (%)	Controls (n = 96) N (%)	P-value	OR (95%CI)
Genotype				
C/C	78 (80.4%)	71 (74%)	0.109	0.824 (0.409-1.657)
C/T	19 (19.6%)	21 (21.9%)		0.101 (0.005-1.913)
T/T	0	4 (4.1%)		
Genotype				
C/C	78 (80.4%)	71 (74%)	0.307	0.692 (0.351-1.362)
C//T+T/T	19 (19.6%)	25 (26%)		
Allele				
C	175 (90.2%)	163 (84.9%)	0.125	0.610 (0.329-1.131)
T	19 (9.8)	29 (15.1%)		

OR, odds ratio; CI, confidence interval.
The data are listed as frequencies (number of cases) and percentages.
P < 0.05 is statistically significant.

HCV infection and its related complications are considered the chief public health challenges in Egypt.[19] Accordingly, all national institutions are directed toward researches addressing all aspects of the disease. In respect to the introductory, we aimed to evaluate the potential relationship between the ApoB-516C/T promoter gene polymorphism and HCV in a cohort of Egyptian patients and estimate the role of LDL and ApoB in the prevention of infection.

According to the products of enzymatic digestion of the PCR amplicon by the restriction enzyme *EarI*, analysis of ApoB −516 genotype distributions among the HCV patients and controls showed higher count of the C/C genotype and higher frequency of C allele of the polymorphism in patients than that in healthy individuals and higher counts of the C/T and T/T genotypes and higher frequency of T allele in the control group than in the HCV patients. However, both differences showed no statistical significance (*P* = 0.109, 0.125, respectively). These findings suggest that there is no association between *ApoB* promoter

ApoB −516C/T gene polymorphism and HCV infection in our patient cohort. Stratification analysis showed that the frequency of the C/T genotype was significantly lower in female patients with HCV infection compared with female controls. This indicates that the presence of the C/T genotype may be a protective factor against HCV in this gender (*P* = 0.011, OR = 0.132, 95%CI = 0.026-0.657).

To the best of our knowledge, the concept of the current study was investigated only once in a previous study carried out by Zhu et al[20] in different ethnic group. However, in contrast to our findings, they reported an association between the ApoB-516C/T promoter gene polymorphism and the susceptibility to HCV infection, with the C/C genotype showing increased risk to the development of the disease (*P* < 0.001). The discrepancies in results could also be attributed to many causes as different ethnicities, environmental factors and the genetic background of different patients in addition to the difference in a number of included subjects in both studies.

TABLE 3 Comparison of genotype count of ApoB −516 polymorphism between HCV infected patients and healthy individuals in both sexes

	Male patients (n = 66)	Male controls (n = 64)	P-value	OR (95%CI)	Female patients (n = 31)	Female controls (n = 32)	P-value	OR (95% CI)
Genotype								
C/C	49 (74.2%)	50 (78.1%)	0.055	1.735 (0.723-4.161)	29 (93.5%)	21 (65.6%)	0.011	
C/T	17 (25.8%)	10 (15.6%)		0.113 (0.006-2.161)	2 (6.5%)	11 (34.4%)		0.132 (0.026-0.657)
T/T	0	4 (6.3%)			0	0		—
Allele								
C	115 (87.1%)	110 (85.9%)	0.857	0.903 (0.443-1.842)	60 (96.8%)	53 (82.8%)	0.016	0.161 (0.034-0.758)
T	17 (12.9%)	17 (14.1%)			2 (3.2%)	11 (17.2%)		

OR, odds ratio; CI, confidence interval.
The data are listed as counts (number of cases) and percentages.
P < 0.05 is statistically significant.

TABLE 4 Comparison between the −516C/T genotypes of *ApoB* gene and serum lipid and ApoB profiles in HCV patients

	Genotype		
	C/C (*n* = 78)	C/T (*n* = 19)	*P*-value
Sex			
Male	49 (62.8%)	17 (89.5%)	0.029
Female	29 (37.2%)	2 (10.5%)	
Age	45.9 ± 5.0	47.6 ± 4.6	0.182
TC (mg/dL)	151.2 ± 21.8	147.9 ± 23.6	0.565
TG (mg/dL)	139.6 ± 28.2	145.4 ± 28.5	0.430
HDL (mg/dL)	35.2 ± 6.3	34.6 ± 5.6	0.715
LDL (mg/dL)	79.1 ± 22.9	80.4 ± 24.0	0.824
ApoB (mg/dL)	67.8 ± 15.4	68.5 ± 12.9	0.865

In this study, we compared the two groups (HCV-infected patients and healthy individuals) as regards the lipid profile data and ApoB level in sera. Results show that the levels of TG, TC, HDL, LDL, and ApoB were significantly lower in patients compared to the control group ($P < 0.001$). In accordance with this are the findings reported by Zhu et al,[20] however, no significant difference in the levels of triglycerides was observed between the two groups in their study.

In addition, we analyzed the effects of the −516C/T polymorphism of *ApoB* gene on serum lipid and Apo B profiles. However, we did not find any statistically significant differences between the C/C and C/T groups of patients in respect to all these levels ($P > 0.05$). Similarly, a study conducted by Perez-Martinez et al[21] suggested that the ApoB-516C/T polymorphism does not affect the lipid profile even after the changes in the dietary fat intake in a healthy population. In contrast,[20] reported higher plasma levels of ApoB and LDL in −516T allele carriers patients with the polymorphic genotypes (C/T and T/T) than homozygous of the −516C allele ($P < 0.001$). This is consistent with the previous finding that the C to T switch in the promoter region at −516 of *ApoB* gene is associated with a major increase in the circulating levels of LDL and ApoB.

Also, our results show that the mean values of LDL and Apo-B in HCV patients were 79.4 ± 23.0 and 67.9 ± 14.9, respectively, compared to healthy controls (137.2 ± 14.7 and 89.6 ± 9.8, respectively) with a highly significant *P*-value ($P < 0.001$). Based that our two groups were almost exposed to the same risk factors, so we assumed that higher levels of LDL and ApoB in healthy controls competed with the LVP and prevented its binding to the LDLR and accordingly, protected the controls from HCV infection. This assumption was also reported in previous studies.[22,23] In contrast, lower levels in patients might be the factor that helped HCV LVP binding to LDLR, entry to the cells and subsequent infection.

5 | CONCLUSIONS

This study exonerated the possible effect of *ApoB* polymorphism on HCV development in our cohort of Egyptian patients. However, our study firstly points to the possibility of the implication of this polymorphism in the susceptibility to the infection in Egyptian females. This finding is limited in term of the low sample size of female patients recruited in the study. Accordingly, further studies with larger female cohorts are needed to verify our results. This step forward could be established as a genetic risk prediction factor for HCV female patients in our country and hence could be implemented in gender-specific medicine. Additionally, we assume that high levels of LDL and ApoB might protect against HCV infection.

DISCLOSURE

The authors have no conflicts of interest to declare.

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