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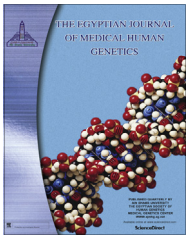

ORIGINAL ARTICLE

Patatin-like phospholipase domain containing-3 gene (PNPLA3) I148M polymorphism and liver damage in chronic hepatitis C Egyptian patients

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Abstract Hepatitis C virus (HCV) has been associated with high prevalence of steatosis and fibrosis. The impact of single-nucleotide polymorphisms (SNP) of patatin-like phospholipase domain-containing protein 3 (PNPLA3) on the development of steatosis and fibrosis is not clarified for Egyptian patients with chronic hepatitis C (CHC).

The aim of the study was to assess whether the PNPLA3 I148M variant predisposes to steatosis, and to progressive liver damage, as evaluated fibrosis stage in Egyptian patients with CHC.

Subjects and methods: 218 CHC Egyptian patients were enrolled in the study, they were grouped by Ishak stage of fibrosis on liver biopsy into group I (fibrosis score 0 or 1; n 81), group II (fibrosis score 2 or 3; n 72) and group III (fibrosis score 4–6; n 65). DNA was amplified by polymerase chain reaction.

Results: PNPLA3 genotype was not associated with metabolic parameters, including body mass index (BMI) and lipid levels, but the presence of G allele is associated with higher liver enzymes and viral loads levels. The PNPLA3 G allele was associated with the severity (stage) of fibrosis (X^2 = 35.83, P = 0.000). Steatosis was detected in 40.7% of C allele carriers and 58.53% of G allele carriers. The rs738409 G allele was significantly associated with steatosis in the overall CHC patients with different fibrotic stages (X^2 = 6.023, P = 0.018) odds ratio (OR) and 95% CI = 1.99 (1.146–3.47).

Conclusion: Steatosis and fibrosis severity seems to be PNPLA3 I148M regulated, independently to metabolic parameters but with significant association with viral loads and liver enzymes values in Egyptian patients with CHC.

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1. Introduction

Chronic hepatitis C infection (CHC) affects more than 170 million individuals worldwide, representing a leading cause of liver-related mortality [1]. CHC encompasses a wide spectrum of diseases, ranging from minimal disease to active hepatitis, which frequently progresses to cirrhosis and hepatocarcinoma [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 [2]. Hepatic stellate cells (HSC) are thought to play a pivotal role in fibrogenesis within the liver [2]. Steatosis accelerates activities and progression of chronic hepatitis C and is independently associated with stage III/IV hepatic fibrosis [3]. Overall sustained virological response to treatment in HCV-infected patients with steatosis is also considerably lower than in those without steatosis [4]. Patatin-like phospholipase domain-containing 3 (PNPLA3) gene, also called adiponutrin, with a molecular mass of approximately 53 kDa that in humans is mainly expressed in intracellular membrane fractions in hepatocytes [5,6] which encodes a 481 amino acid protein, is a triacylglycerol lipase conserved from potatoes to humans with 10-fold higher expression in liver compared to adipose tissue [5]. Variation in PNPLA3 gene contributes to ethnic and inter-individual differences in hepatic fat content and susceptibility to nonalcoholic fatty liver disease (NAFLD) [6]. It was reported that the minor allele of rs738409 C/G, a nonsynonymous coding single nucleotide polymorphism (SNP) in the PNPLA3 gene encoding I148M change, was associated with steatosis, portal inflammation, lobular inflammation, Mallory-Denk bodies, NAFLD activity score (NAS) and fibrosis [7]. Minor allele of rs738409 C/G is also strongly associated with hepatic fat content and with elevated serum levels of ALT and AST [8]. Toll-like receptor 4 (TLR4) is a receptor for bacterial lipopolysaccharide (LPS), which is suggested to be involved in the pathogenesis of hepatobiliary diseases [9]. There is accumulating evidence that LPS and TLR4 play a key role in the pathogenesis of HCV infection. Patients with chronic HCV infection display increased serum levels of LPS even in the absence of significant hepatic fibrosis [10].

The mechanism whereby rs738409 influences liver fat is independent of body composition and insulin resistance [11,12], but likely involves a decreased ability of the 148M PNPLA3 variant to regulate hepatic lipid metabolism [13]. However, it is not known whether the rs738409 SNP influences the steatogenic effect of HCV and the progression of CHC in Egyptian patients. Although most lines of evidence support a strong association between steatosis and fibrosis severity in CHC [14], doubts have been molded as to whether steatosis is the causative factor driving accelerated hepatic fibrogenesis, or rather is a simple marker associated with increased fat stores which would represent the occurrence of underlying disease progression [15]. This study was designed to assess whether the PNPLA3 I148M variant predisposes to steatosis, and to progressive liver damage, as evaluated fibrosis stage in Egyptian patients with CHC. To explore the possible clinical relevance of these findings, we also assessed the effect of PNPLA3 genotype on metabolic parameters such as lipid levels, viral load values and liver enzymes in CHC.

2. Subjects and methods

This study was carried out in the Medical Biochemistry and Molecular Biology and Internal Medicine, Pathology Departments, Faculty of Medicine, Outpatients Clinics, Intensive Care Unit of Zagazig University Hospitals and Private outpatient Clinics, Zagazig City during the period from Nov 2013 to April 2015. In this study we examined the effect and relationship of allelic variants of the *PNPLA3* rs738409 C > G gene in Egyptian patients with chronic HCV infection with steatosis and different degree of liver fibrosis according to Ishak classification [16]. PCR technique with restriction fragment length polymorphism (RFLP) was used to detect the gene polymorphism.

2.1. Study subjects

Two hundred and eighteen of chronic HCV infection subjects (113 females, 105 males) were included in this study. They did not receive any treatment of hepatitis C virus before the study. They were diagnosed by clinical, laboratory and ultrasonography as HCV infected patients. HCV infection was diagnosed as +ve enzyme linked immunosorbent assay technique (ELISA) for anti HCV antibodies. The presence of anti-HCV antibodies was determined in serum samples by ELISA. Moreover, the HCV infection was detected by +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, tissue sections were stained with hematoxylin and eosin, impregnated with silver for reticulin framework, and stained with trichrome for collagen. They were graded for the degree of necro-inflammatory activity and staged for the extent of fibrosis according to the criteria of Ishak classification [16]. The degree of steatosis was graded as follows on the basis of the percentage of hepatocytes containing macrovesicular fat droplets: grade 0, no steatosis; grade 1, <33% hepatocytes containing macrovesicular fat droplets; grade 2, 33–66% of hepatocytes containing macrovesicular fat droplets; grade 3, >66% of hepatocytes containing macrovesicular fat droplets.

Diagnosis of steatosis by Ultrasonography was based on evidence of ultrasonographic contrast between the hepatic and right renal parenchyma of the right intercostal sonogram in the midaxillary line, or abnormally intense, high-level echoes arising from the hepatic parenchyma, and was graded according to intensity on a three-grade scale as none, mild, or severe [17]. 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, the work has been carried out in accordance with the code of ethics of the world medical association (Declaration of Helsinki) for experiments in humans. Exclusion criteria were positive hepatitis B viral markers, 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) [18]. Liver disease associated with drug use and primary biliary cirrhosis that can be diagnosed by symptoms

Table 1 Basic characteristics of all participant groups.

Parameters	Group I (n = 81)	Group II (n = 72)	Group III (n = 65)	Anova (F) value with P values
Age (years)	48.16 ± 4.67	47.50 ± 4.77	49.34 ± 5.87	F = 1.851, P = 0.104
BMI (kg/m^2)	24.95 ± 4.28	25.75 ± 4.91	26.41 ± 4.61	F = 2.18/, P = 0.134
TC (mg/dl)	217.29 ± 34.24	226.28 ± 20.1	218.96 ± 20.53	F = 4.13,P = 0.105
TG (mg/dl)	154.6 ± 21.3	153.4 ± 24.0	160.89 ± 25.12	F = 3.43, P = 0.101
Serum ALT (IU/L)	93.17 ± 23.44	178.61 ± 43.63	193.57 ± 27.25	F = 210.315, P = 0.000*
Serum AST (IU/L)	118.57 ± 43.24	174.88 ± 44.3	175.42 ± 48.65	F = 39.750, P = 0.000*
Serum GGT (IU/L)	73.41 ± 20.19	109.48 ± 14.02	118.94 ± 11.55	F = 169.196, P = 0.000*
Viral load Eq/ml	$1\times10^6 \pm 3.66\times10^5$	$229.96\times10^3 \pm 143\times10^3$	$854.08\times10^3 \pm 473.41\times10^3$	F = 152.270, P = 0.000*
Serum creatinine (mg/dl)	0.97 ± 0.25	0.99 ± 0.24	1.01 ± 0.21	F = 0.41, P = 0.664
Serum urea (mg/dl)	38.18 ± 5.63	39.9 ± 4.78	38.25 ± 6.11	F = 2.47, P = 0.087

N.B. P^* signs for significant P value.

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 [18]. Patients lacked a liver biopsy, the histological sample was not adequate, or had coexistent liver diseases were excluded from the study, Informed consent was obtained from all patients before participation in this study. The clinical features of the subjects are summarized in Table 1.

2.2. 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 PNPLA3 rs738409 gene polymorphism determination and the other serum sample for estimation of triglyceride (TG), total cholesterol (TC), urea, creatinine, other liver function test (LFT) parameters (ALT, AST, GGT) and viral load. They were measured by standard chemical and enzymatic commercial methods in the Medical Biochemistry Department, Zagazig University.

2.3. Methods

All subjects are submitted to the following:

(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:
      – Triglyceride (TG) and total cholesterol (TC) were estimated by enzymatic method [19].
      – Measurement of serum urea and creatinine standard chemical and enzymatic commercial methods.
      – Anthropometric examination (height and weight) for calculation of the BMI was done for all the subjects (kg/m^2).
      – Liver function tests:
      – Serum alanine transaminase (ALT), aspartate transaminase (AST) and gamma glutamyl transferase (GGT) using bio-Merieux kit [20].
      – 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 [16]:
         (1) The statement that it is, indeed, presence of 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.

2.4. DNA preparation and SNP genotyping

The rs738409 C > G SNP, encoding I148M, was genotyped by the polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) method according to Islek et al. [21].

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 PCR was performed in a Perkin Elmer 4800 thermal cycler (PTC-100 machine, Inc., Watertown, Mass. USA). PCR was performed with PCR GTX press Master Mix (Applied Biosystems) as following: 25 μL of PCR master mix was dispensed into each PCR tube, and then the following materials were added to each tube containing 100 ng of extracted DNA, 25 μM forward primer (5′-CCCTGCTCACT TGGAGAAAG-3′), and 25 μM reverse primer (5′ – TGTCA CCGGAATAGGGAGGA-3′) (Operon Biotechnologies, Inc.), and then 19 μL dd H2O was added giving a final volume of 50 μL.

The PCR cycling conditions for the rs738409 variant were as follows: briefly, the genomic DNA was denatured at 95 °C for 5 min, followed by 35 cycles at 95 °C for 1 min, 58 °C for 30 s, 72 °C for 1 min, and a final extension step at 72 °C for

10 min. The digestion of the amplified 139 bp fragment with the restriction endonuclease *NlaIII* site at the position of 44324727 bp was carried out at 37 °C overnight. The digested PCR products were resolved on 3% agarose gels stained with ethidium bromide. PCR method resulted in a 139 bp product of CC genotype and the GG genotype showed two fragments of 112 and 27 bp. In heterozygous genotype samples (G/C) showed three fragments of 139, 112, and 27 bp.

3. Statistical analysis

Results were statistically analyzed using SPSS version 16 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 subjects in all studied groups within group comparisons. Post Hoc Tukey HSD (high significance difference) calculates a new critical value that can be used to evaluate whether differences between any two pairs of means are significant. Odds ratio (OR) and 95% confidence interval (CI) for the presence of HCV infection and steatosis with different liver fibrosis grades within the PNLP genotypes were analyzed by using the chi square X^2 test. Results were considered significant when $P < 0.05$.

4. Results

Two hundred eighteen chronic HCV infected patients (113 females, 105 males with mean ages of 46.44 ± 6.06 years were evaluated in this study. Anova test (*F*-test) revealed a non-significant difference between groups regarding sex and age ($F = 0.976$, $P = 0.104$), ANOVA test (*F*-test) of different measured parameters, Table 1.

4.1. Fibrosis scores

Ishak classification classified the patients according to the stage of liver fibrosis on liver biopsy into three groups as follows:

- Group I: "HCV with fibrosis score 0 or 1": This group comprised 81 patients (37.15%) (44 females and 37 males) with ages ranging between 36 and 56 years with a mean value ± S.D of 48.16 ± 4.67 years. Steatosis was detected in 30 patients (37.03%).
- Group II: "HCV with fibrosis score 2 or 3": 72 patients (33.02%) (40 females and 32 males) were included with ages ranged from 38 to 57 years with a mean value ± S.D of 47.5 ± 4.77 years. 44 patients (61.11%) had steatosis.
- Group III: "HCV with fibrosis score 4 or 6": 65 patients (29.81%) (29 females and 36 males) with ages ranging between 36 and 61 years with a mean value ± S.D of 49.34 ± 5.9 years. Steatosis was reported in 37 patients (56.92%).

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

4.2. Distribution of PNPLA3 gene polymorphism in all patient groups

The frequency of PNPLA3 rs738409 genotypes is summarized in Table 2 and Fig. 2. The frequency of C and G allele in total patients with chronic HCV infection was 271(62.15%) and 165 (37.85%), respectively.

- In group I: The homozygous CC was detected in 58 patients (71.6%), the heterozygous CG in 14 patients (17.28%) and the homozygous GG in 9 patients (11.11%). The C and G allele frequency were 80.25% and 19.75%, respectively.
- In group II: There were 31 patients (43.1%) of homozygous CC genotype and there were 28 patients with CG genotype (38.8%), we found only 13 (18.1%) patients carrying the homozygous GG genotype. The C and G allele frequency were 62.5% and 37.5%, respectively.
- In group III: The PNPLA3 homozygous CC, heterozygous CG and homozygous GG genotypes were detected in 14 patients (21.54%), 23 patients (35.38%) and 28 patients (43.1%), respectively. The C and G allele frequency were 39.2% and 60.8%, respectively.

We revealed a significant association with PNPLA3 gene (C/G) polymorphism among chronic HCV infected patients with different fibrosis degrees ($X^2 = 35.83$, $P = 0.000$) (Table 2).

The GG genotype and the G allele for the *PNPLA3* rs738409 were significantly more frequent in group III than in group II and group I patients ($X^2 = 12.074$, $P = 0.002$ and $X^2 = 37.532$, $P = 0.000$), ($X^2 = 30.46$, $X^2 = 51.49$, $P = 0.000$), odds ratio (OR) and 95% CI for the G allele = 4.35 (2.55–7.44) and 6.3 (3.73–10.61), respectively. Moreover, a significant increase of GG genotype and G allele

Table 2 Genotype and Allele frequency for *PNPLA3* rs738409 gene polymorphism in all studied groups.

C/G			
Genotype	Groups		
	Group I ($N = 81$) *N* (%)	Group II ($N = 72$) *N* (%)	Group III ($N = 65$) *N* (%)
CC	58 (71.6%)	31 (43.1%)	14 (21.53%)
CG	14 (17.28%)	28 (38.8%)	23 (35.38%)
GG	9 (11.11%)	13 (18.1%)	28 (43.1%)
X^2// *P* value	7.02^a// $P = 0.03^{a,*}$ 37.532^b// $P = 0.000^{b,*}$ 12.07^c// *P* value = $0.002^{c,*}$		
	n (%)	*n* (%)	*n* (%)
C allele	130 (80.25%)	90 (62.5%)	51 (39.20%)
G allele	32 (19.75%)	54 (37.5%)	79 (60.80%)
X^2	11.883^a	51.493^b	30.46^c
P	$0.001^{a,*}$	$0.000^{b,*}$	$0.000^{c,*}$
OR 95%CI	2.43 (1.45–4.07)	6.29 (3.73–10.61)	4.35 (2.55–7.44)

Comparison of Group I and Group II[a], Group I and Group III[b], Group II and Group III[c], P^* points for significant *P* value.

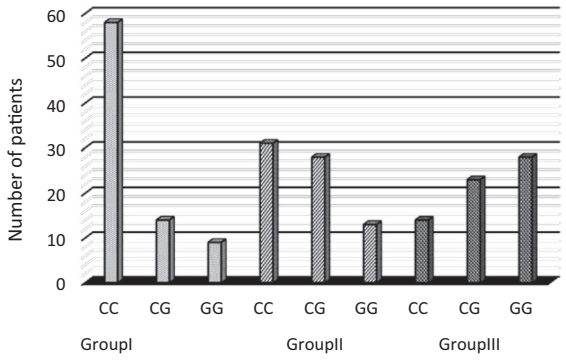

Figure 1 Frequency of different PNPLA3 genotypes in all studied groups.

frequency was observed in group II more than group I patients (X^2 = 7.02, P = 0.03, $X^{2\ =\ 11.88,\ P}$ = 0.001) odds ratio (OR) and 95% CI for the G allele = 2.43 (1.46–4.07) (see Fig. 1).

4.3. Association between rs738409 genotypes and hepatic fibrosis stage with or without hepatic steatosis

We investigated the association between rs738409 genotypes with hepatic fibrosis stage and the presence or absence of hepatic steatosis. The results of the analysis revealed an additive increase in the fibrosis severity and occurrence of steatosis in the rs738409 G-allele harboring patients. The prevalence of steatosis according to rs738409 gene C and G alleles is shown in Fig. 2 and Table 3. Steatosis was detected in 37.5% and 37% of group I G allele and C allele carriers, respectively. In group II, 67% of G allele carriers and 58% of C allele carriers were recorded to have steatosis, while steatosis was proved in 60% of G allele carriers and 52% of C allele carriers in group III patients. The results showed that presence of steatosis was significantly increased in the G allele carriers relative to the C allele carriers in all groups (X^2 = 34.746, P = 0.000), Table 3.

In overall studied patients, steatosis was detected in 55 patients out of 135 patients of C allele carriers (40.7%) and 48 patients out of 82 patients of G allele carriers (58.53%). The rs738409 G allele was significantly associated with steatosis in the overall CHC patients with different fibrotic stage (X^2 = 6.023, P = 0.018) odds ratio (OR) and 95% CI for the G allele = 1.99 (1.146–3.47). The PNPLA3 rs738409 genotype was significantly associated with Ishak fibrosis stage, Homozygosity for the rs738409 G allele was significantly more represented in CHC patients with than in those without steatosis, and the association was independent of age, sex, BMI and lipid levels.

4.4. Data of liver function test (LFT) and other metabolic parameters (Table 1)

On comparing ALT, AST and GGT serum levels among CHC patients groups, there was a significant increase in group II and group III than group I patients (Tukey HSD = 85.44, 100.39, 56.311,56.84, 36,07, 45.52, P = 0.000), respectively. ALT and

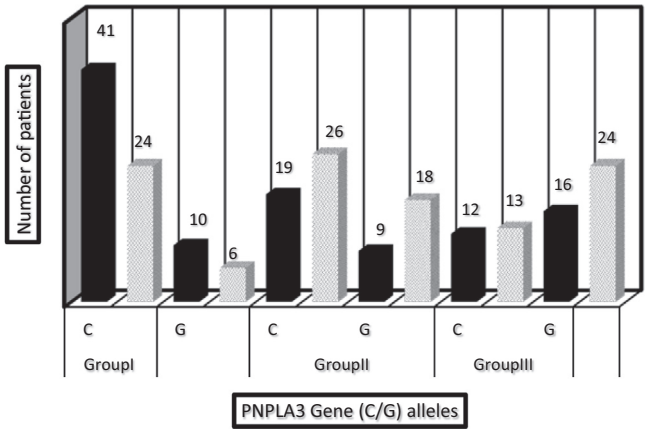

Figure 2 Number of HCV patients with and without steatosis according to PNPLA3 alleles (C and G) for each group. (N.B. Plain solid columns for the patients without steatosis and the dotted ones for the patients with steatosis).

Table 3 Number of HCV patients with and without steatosis according to PNPLA3 alleles (C and G) for each group.

Patients	Group I (N = 81)		Group II (N = 72)		Group III (N = 65)	
	C allele N = 65	G allele N = 16	C allele N = 45	G allele N = 27	C allele N = 25	G allele N = 40
Without steatosis	41 (63%)	10 (62.5%)	19 (42%)	9 (33%)	12 (48%)	16 (40%)
With steatosis	24 (37%)	6 (37.5%)	26 (58%)	18 (67%)	13 (52%)	24 (60%)
X^2 and P value	X^2 = 13.17, P = 0.003[a] *					
	X^2 = 29.92, P = 0. 000[b] *					
	X^2 = 8.76, P = 0.033[c] *					

Comparison of Group I and Group II[a], Group I and Group III[b], Group II and Group III[c], P* points for significant P value.

GGT showed significant increase in group III more than group I and II patients (Tukey HSD = 14.95, $P = 0.02$) (Tukey HSD = 9.45, $P = 0.002$), respectively, with non-significant association in AST serum between group II and III patients (Tukey HSD = −0.53, $P = 0.997$). Viral load values recorded a significant increase in group III and group II patients more when compared to group I with significant increase in subgroup III more than subgroup II patients (Tukey HSD = 7.45, 1.3 ($P = 0.01$) and 6.24, $P = 0.000$), respectively.

4.5. Association of PNPLA3 gene genotype with liver enzymes and other metabolic parameters

In addition, we also compared the *PNPLA3* rs738409 genotypes with different clinical parameters of all studied patients. In CHC patients the *PNPLA3* rs738409 genotype was not significantly associated with age, sex distribution, BMI, total cholesterol (TC) and TG. Otherwise, serum AST, ALT and GGT levels were significantly higher in patients harboring the G allele. Viral load values (Eq/ml) recorded significant differences among different PNPLA3 rs738409 genotypes of all CHC patients to be increased in patients harboring the GG and G allele, listed in Table 4.

5. Discussion

Since the discovery of hepatitis C, there have been several investigations of the role of histological biopsy in determining the severity of chronic hepatitis and progression of disease [22]. Most of these studies used histological scoring systems for chronic hepatitis that did not include the assessment of steatosis [23]. The extent of fibrosis and architectural disturbance (stage of disease) increases with time, but at a rate that varies widely between individuals, with some progressing to cirrhosis within 10 years of viral acquisition, and others showing only mild fibrosis after 30 years [24]. Steatosis is seen in 30–70% of liver biopsies from patients with chronic hepatitis C [25,26], more frequently than is seen in other causes of chronic hepatitis [27], and in a high proportion of patients no other cause of fatty liver, such as high alcohol intake or high BMI can be identified [28]. Steatosis is thought to be a specific cytopathic effect of the hepatitis C virus (HCV) and favors hepatitis C virus (HCV) life-cycle [29], and may be particularly associated with the type 3 genotype [30].

Despite being initial hypothesis-driven, case-control studies identified some genetic loci associated with the progression of liver damage, the genetic determinants of NAFLD remained obscure until recently [31]. By 2008, the first genome wide association studies in the field of hepatic steatosis allowed to identify the rs738409 variant, by a hypothesis free drive approach, as the single major genetic determinant of hepatic fat content. This sequence variation is a C > G single nucleotide change, encoding for the 148 Isoleucine to Methionine protein variant (I148M) of Patatin-like phospholipase domain-containing 3 (PNPLA3) [6].

To our knowledge, there are few studies which discussed the influence of rs738409 PNPLA3 SNP, on the risk of pathogenesis of steatosis and fibrosis progression in CHC Egyptian patients. Thus, our study was designed to examine the role of rs738409 C/G genotypes on histologically assessed liver

Table 4 Different Studied parameters and *PNPLA3* rs738409 gene polymorphisms in different groups.

Parameters	Groups genotypes								
	Group I			Group II			Group III		
	CC $N = 58$	CG $N = 14$	GG $N = 9$	CC $N = 31$	CG $N = 28$	GG $N = 13$	CC $N = 14$	CG $N = 23$	GG $N = 28$
BMI (kg/m^2)	22.02 ± 2.5	21.75 ± 3.3	23.35 ± 6.6	26.7 ± 5.3	28.1 ± 4.8	26.9 ± 4.6	26.04 ± 6.3	24.6 ± 5.9	25.4 ± 4.22
	$F = 0.729$, $P = 0.49$			$F = 0.641$, $P = 0.53$			$F = 0.345$, $P = 0.710$		
TC (mg/dl)	215.2 ± 29.2	223.1 ± 54.9	222.04 ± 25.5	225.8 ± 24.32	227.3 ± 21.4	241.9 ± 8.58	217.4 ± 26.6	213.3 ± 12.7	224.4 ± 21.7
	$F = 0.393$, $P = 0.676$			$F = 2.828$, $P = 0.066$			$F = 1.931$, $P = 0.154$		
TG (mg/dl)	142.7 ± 16.6	140.88 ± 11.12	149.43 ± 30.3	155.5 ± 27.7	173.13 ± 33.02	165.5 ± 36.11	190.4 ± 29.9	181.1 ± 32.92	181.99 ± 41.6
	$F = 0.692$, $P = 0.503$			$F = 2.341$, $P = 0.104$			$F = 0.325$, $P = 0.723$		
ALT (IU/L)	85.1 ± 22.4	112.7 ± 10.0	114.7 ± 9.4	148.59 ± 46.56	207.86 ± 23.05	189.83 ± 17.13	168.4 ± 22.7	193.4 ± 24.7	206.3 ± 22.8
	$F = 16.91$, $P = 0.000^*$			$F = 21.523$, $P = 0.000^*$			$F = 12.22$, $P = 0.000^*$		
AST (IU/L)	106.5 ± 34.6	153.3 ± 50.5	142.2 ± 47.6	146.2 ± 44.75	186.32 ± 29.7	218.7 ± 11.7	186.5 ± 29.34	214.9 ± 17.8	137.5 ± 45.4
	$F = 9.88$, $P = 0.000^*$			$F = 21.932$, $P = 0.000^*$			$F = 32.7$, $P = 0.000^*$		
GGT (IU/L)	67.56 ± 18.2	89.6 ± 18.5	85.96 ± 17.0	103.1 ± 11.66	109.43 ± 14.15	124.8 ± 3.6	110.2 ± 12.2	120.3 ± 10.73	122.21 ± 9.94
	$F = 10.76$, $P = 0.000^*$			$F = 15.416$, $P = 0.000^*$			$F = 5.108$, $P = 0.004^*$		
Viral load values Eq/ml	88.8×10^3 ± 34.8×10^3	123.7×10^3 ± 26.5×10^3	135.7×10^3 ± 17.7×10^3	288.3×10^3 ± 133×10^3	163.18×10^3 ± 128.0×10^3	234.7×10^3 ± 146.4×10^3	607.1×10^3 ± 31.97×10^3	812.2×10^3 ± 499. 96×10^3	101.2×10^4 ± 46.83×10^3
	$F = 12.86$, $P = 0.000^*$			$F = 6.439$, $P = 0.003^*$			$F = 3.87$, $P = 0.026^*$		

Anova (F) test to examine the different Studied parameters with *PNPLA3* rs738409 gene polymorphisms in different groups. P^* refers for significant P value.

steatosis and the severity of liver fibrosis and to explore the possible clinical relevance of these findings, we also assessed the effect of PNPLA3 genotype on metabolic parameters such as lipid profile and BMI, viral loads and liver enzymes in CHC.

We evaluated 218 of Egyptian CHC patients, the patients were classified according to fibrosis stage by Ishak classifications into three groups as 37.15% (Ishak = 0–1), 33.02% (Ishak = 2–3) and 29.81% (Ishak = 4–6). Steatosis has been detected in group I, group II and group III as follows 37.03%, 61.11% and 56.92%, respectively. We recorded positive association between occurrence of steatosis and the stage of fibrosis in HCV patients.

The presence of steatosis in patients with hepatitis C is dependant on a complex interaction of viral and host related factors [29]. Steatosis in patients without hepatitis C is related to alcohol consumption, obesity, high BMI, type II diabetes, and hyperlipidaemia [32]. These factors are also important in patients with hepatitis C, but a proportion of patients with hepatitis C have no other risk factor for steatosis. In particular, this has been reported to be a feature of genotype 3 infection, so that patients with moderate to severe steatosis without other risk factors are probably infected with genotype 3 [28]. This interacts with factors predisposing to steatosis in the host, particularly high BMI, to result in the expression of steatosis in a proportion of HCV positive patients [33]. An evidence that weight reduction in patients who are mildly overweight results is improvement in steatosis, and in some also in the degree of fibrosis [33], suggests both the reversibility of steatosis in patients with chronic hepatitis C, and also the direct importance of steatosis in the development of fibrosis.

Nishina et al. [34] observed fat accumulation in the liver in transgenic mice expressing HCV polyprotein and reported that iron-induced unfolded protein response appeared to be one of the mechanisms responsible for hepatic steatosis in HCV infection. HCV particles were observed in close proximity to lipid droplets, an organelle used for the storage of neutral lipids that moves dynamically through the cytoplasm, interacting with other organelles, including the endoplasmic reticulum [35]. These findings indicate that some steps of HCV assembly take place around lipid droplets [35], suggesting that this might be a possible mechanism for HCV directly inducing hepatic steatosis.

PNPLA3 rs738409 SNP has been previously reported to influence liver fat without affecting body composition and insulin resistance and has been concluded that this genetic variant affects steatosis development and fibrosis stage in CHC patients [36,37].

We evaluated the rs738409 PNPLA3 SNP In CHC Egyptian patients, and observed that this genetic variant of PNPLA3 gene (C/G) polymorphism affects steatosis development and is independently associated with fibrosis severity among chronic HCV infected patients with different fibrosis degrees. In line with the prediction that, if steatosis causes fibrosis progression in CHC, then the rs738409 SNP C/G alleles should also be associated with advanced fibrosis. In the present study, steatosis has been detected in 37% of G allele carriers and 36.9% of C allele carriers in group I patients. In group II, 66.66% of G allele carriers and 57.77% of C allele carriers were recorded to have steatosis, while steatosis was proved in 60% of G allele carriers and 52% of C allele carriers in group III patients. Steatosis was detected in 55 patients out of 135 patients of C allele carriers (40.7%) and 48 patients out of 82 patients of G allele carriers (58.53%) in overall CHC patients. The rs738409 G allele was significantly associated with steatosis in the overall CHC patients with different fibrotic stages.

Moreover, the association between rs738409 genotype and steatosis and fibrosis was independent of known risk factors, such as age, sex, BMI, TC and TG, but we observed a significant association with liver enzymes such as ALT, AST and GGT levels and viral loads. Homozygosity for the minor rs738409 G allele conferred the strongest risk of steatosis and fibrosis severity.

Our results were in harmony with Valenti et al. [38] and Romeo et al. [6] who reported that homozygosity for the minor rs738409 G allele conferred the strongest risk of steatosis.

Valenti et al. [36] speculated that when steatosis inducing stressors such as obesity and insulin resistance, excess alcohol intake, and HCV infection stress the liver, in the presence of the "normal" 148I PNPLA3 allele the damage will result in simple uncomplicated steatosis, whereas the 148M "at risk" allele will favor steatohepatitis and fibrogenesis, with progression toward cirrhosis and its complication in susceptible individuals.

These findings were in concordance also with Dongiovanni et al. [39], Ampuero et al. [40] and Amit et al. [41] who stated that PNPLA3 allele-G modulated the development of steatosis, particularly in patients with HCV.

Association of rs738409 with ALT and AST levels is controversial. In Argentinians with NAFLD [42], Italian adults with NAFLD [25], obese Italian adults [38], and obese Italian children [43], the G-allele of rs738409 was found to be significantly related to increased ALT levels. In Finnish individuals [44], obese Italian adults, the G-allele of rs738409 is significantly associated with increased AST levels [39]. However, in the African American, European American [6], and German [37] populations, rs738409 is not associated with either ALT or AST levels.

Hotta et al. [45] found that in the Japanese population, the PNPLA3 rs738409 was associated with plasma levels of ALT, AST, and the histological fibrosis stage. They suggested that PNPLA3 may be involved in the progression of fibrosis.

Valenti et al. [15] stated that PNPLA3 genotype influences steatosis development in CHC, is independently associated with cirrhosis and other steatosis-related clinical outcomes, such as lack of response to antiviral treatment and HCC.

Kitamoto et al. [46] demonstrated that the PNPLA3 gene is strongly associated with the progression of nonalcoholic steatohepatitis (NASH) in Japanese, whose BMI is lower than that of the United States and European countries [47].

It was reported that the minor allele of rs4986791 C 1196C > T, a non-synonymous coding SNP in the Toll like receptor 4 (TLR4) gene encoding T399I change, emerged as conferring protection against fibrosis progression compared to a major, wild-type (WT) CC allele, along with another highly consegregated SNP (rs4986790, C 896A > G) located at codon position 299 (p.D299G) [48]. These TLR4 SNPs are associated with protection against hepatic fibrosis, reduce TLR4-mediated inflammatory and fibrogenic signaling, and lower the apoptotic threshold of activated hepatic stellate cells [49] TLR4 SNPs also modulate the risk of liver fibrosis in Caucasians with chronic hepatitis C infection [50]. This genetic variant not only influences the overall amount of

hepatocellular fat, but by impacting on the concentration or subcellular localization of specific lipid species directly modulates inflammation. It should not be forgotten that several lipids behave as inflammatory mediators acting through specific receptors [51]. Alternatively, if the 148M mutation slows down triglycerides kinetics between cell compartments, it could be speculated that renders them more susceptible to lipoperoxidation, leading to oxidative stress, and in turn to hepatocellular damage and inflammation [51].

In conclusion, our results showed association between PNPLA3 rs738409 and hepatic steatosis and liver fibrosis severity independently to lipid profile and BMI with significant association with viral loads and liver enzymes, steatosis and fibrosis severity seems to be PNPLA3 regulated in Egyptian patients with CHC.

It is possible that the different evaluation methods, such as Ultrasound and liver biopsy, and different distribution of PNPLA3 rs738409 genotypes might have different association with hepatic steatosis and liver fibrosis in Egyptian patients with chronic hepatitis C. We claim that our study might not be considered ideal to assess the influence of rs738409 genotype on progression of steatosis and fibrosis in HCV. We also could not completely rule out the possibility that there exist different mechanisms of hepatic steatosis and liver fibrosis and hepatitis C. Actually the different therapeutic regimens and HCV genotypes, coupled with the fact that the study was limited to patients who had undergone a liver biopsy, might have accounted for the this study limitations which calls for external validation of our data. Moreover, we conducted this study on a selected group of Egyptian patients and therefore it is not known whether these findings also apply to individuals in the general community with CHC and to patients of different ethnicity. Thus, clarification of the pathogenesis of liver damage progression in CHC, the association between PNPLA3 rs738409 GG genotype and steatosis-related clinical outcomes might identify a subgroup of CHC patients with an adverse prognosis. Whether evaluation of rs738409 genotype can help for treatment and follow-up of CHC patients should be assessed in prospective further studies.

Conflict of interest

The authors declare that there are no conflicts of interest.

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