Original Research Article

Association of Adiponutrin/Patatin Like Phospholipase 3 (PNPLA3) I148M Gene Variant with Chronic Hepatitis C in Egyptian Children

Abstract

Background: Chronic hepatitis C (CHC) represents a leading cause of liver-related mortality worldwide. The patatin-like phospholipase domain-containing 3 gene (PNPLA3/adiponutrin) rs738409 (I148M) single-nucleotide polymorphism (SNP) has been reported to be linked with the severity and progression of liver fat content and liver fibrosis in CHC among different racial groups. Such reports are lacking in CHC Egyptian children.

Aim of study: To evaluate the possible association of PNPLA3-I148M gene variant with the severity of liver fat content and liver fibrosis in Egyptian children with CHC.

Patients and Methods : Fifty normal-weighted children (mean age 10.62±2.59 years) with CHC were subjected to genotyping of PNPLA3-I148M gene variant using the real time PCR TaqMan assay. FibroScan examination for assessment of both liver fibrosis by Fibroscan liver stiffness (LMS) and liver steatosis by the controlled attenuation parameter (CAP) scores and histological examination of liver biopsies for assessment of liver steatosis, and METAVIER scoring for necroinflammatory activity grades and liver fibrosis stages were done for all patients as well as appropriate laboratory investigations. APRI and FIB-4 indices as well as insulin resistance using HOMA-IR were also calculated.

Results: 34 cases (68%) had CC genotype (wild CC genotype), 9 cases (18%) had CG genotype (heterozygous for the risk G allele) and 7 cases (14%) had GG genotype (homozygous for the risk G allele). Significant higher values of LSM and CAP steatosis scores were found in patients with CG and GG genotypes compared to those with CC genotype. CG gene variant and GG gene variant were significant positive predictive factors for histopathological liver steatosis and fibrosis stages and inflammatory activity grades among the studied patients. Significant higher values of many laboratory variables (AST, fasting blood glucose, fasting serum insulin, and HOMA-IR) but lower platelets count was found in patients with G allele (CG+ GG) compared to patients without G allele (CC). In addition, significant positive correlations between PNPLA3-I148M gene variant and indicators of hepatic steatosis and fibrosis and many laboratory parameters (AST, APRI, FIB4, FBS, fasting serum insulin, and HOMA-IR) but a significant negative correlation between PNPLA3-I148M gene variant and platelet cell count were found among the studied patients.

Conclusion: Data of this study suggested that polymorphisms in the PNPLA3 I148M gene variant could contribute to the severity of hepatic steatosis and fibrosis of the studied Egyptian children with CHC.

Keywords: *Chronic Hepatitis C, Liver fibrosis, Steatosis, Patatin Like Phospholipase 3 (PNPLA3) I148M Gene*

Introduction

Chronic hepatitis C (CHC) encompasses a wide spectrum of diseases, ranging from minimal disease to active hepatitis, which frequently progresses to cirrhosis and hepatocellular carcinoma. It represents a leading cause of liver-related mortality worldwide [(1)]. Although the prevalence of CHC is lower in children than adults, an estimated 3.5 to 5 million children worldwide have CHC viral infection [(2, 3)].

Liver steatosis occurs in more than half of hepatitis C (HCV) patients, and has been associated with more aggressive histological features, faster progression of fibrosis, and poorer response to therapy [(4-8)]. Doubts have been cast as to whether steatosis is the causative factor driving accelerated hepatic fibrogenesis, or rather is a simple marker associated with increased fat stores and insulin resistance (IR), which would represent the culprits underlying disease progression [(9,10)].

Both viral and host factors are believed to contribute to CHC-related steatosis [(4, 11, 12)]. A direct cytopathic effect of HCV has been proposed based on the higher prevalence of steatosis in CHC than in other liver diseases, a specific association with genotype 3 infection, and the induction of steatosis by viral proteins [(4, 7, 11-14)].

Genetic host factors have been hypothesized to influence steatosis development and IR in CHC [(15, 16)]. The adiponutrin/patatin-like phospholipase domain-containing 3 (PNPLA3) rs738409 C>G single nucleotide polymorphism (SNP), encoding for the I148M protein variant, has been recognized as a genetic determinant of liver fat content [(17, 18)], and to influence fibrosis severity in patients with Non-alcoholic fatty liver disease (NAFLD) [(19, 20)]. Similarly, in patients with HCV infection, homozygotes for the PNPLA3 I148M has been reported to be associated with more steatosis, fibrosis, cirrhosis, and hepatocellular carcinoma predominantly in adult Mediterranean populations and also, it either negatively impacts therapeutic outcome or is not independently associated with treatment failure [(21-25)].

The mechanism whereby rs738409 influences liver fat is independent of body composition and IR [(17, 18)], but likely involves a decreased ability of the I148M PNPLA3 variant to regulate hepatic lipid metabolism [(26)].The critical amino acid change of isoleucine to methionine at residue 148 has been proposed to result in reduced enzymatic hydrolyses of glycerol lipids, that subsequently leads to induction of steatosis. An alternative hypothesized mechanism of action is that the substitution entails acyl-transferase activity leading to increased triglyceride synthesis [(27)]. The PNPLA3 protein has lipase activity towards triglycerides in hepatocytes and retinyl esters in hepatic stellate cells (HSCs) and the I148M substitution leads to a loss of function [(28)].

Host genetics, for example, IL28B and PNPLA3, impact on direct acting antiviral (DAA)-based treatment for HCV has diminished, but in the setting of shorter duration they appear to influence outcome following otherwise highly effective interferon-sparing regimens. They will remain significant for prediction of the natural course of HCV-related liver disease and may continue to be of importance for tailoring therapy, especially with regards to duration and possible benefit of the addition of ribavirin [(29)].

Data on the relationship between PNPLA3 rs738409 polymorphism and CHC in Egyptian children are lacking. Therefore, our aim in this study was to evaluate the association of this gene variant with the severity of liver fat content and liver fibrosis as assessed by histopathology and by FibroScan with the controlled attenuation parameter (CAP) and to explore the possible clinical relevance of these findings in those children.

Patients and Methods

This was a cross-sectional study, included 50 normal-weight children and adolescents with CHC with Body mass index (BMI) below 85 percentiles based on the national reference data [30]. The study was approved by the Ethical Committee of Faculty of Medicine, Tanta University and written informed consents were obtained from the care givers of all patients.

Exclusion criteria:

Patients with other coexistent etiologies of chronic liver disease as: Hepatitis B, Human immunodeficiency virus (HIV) infection, Autoimmune hepatitis, drug-induced liver injury, or metabolic (Hereditary hemochromatosis, Alpha1-antitrypsin deficiency, Galactosemia, Wilson disease, etc.) and patients with any of the following conditions were excluded:

- Ascites or encephalopathy
- Children less than 3 years old
- Obese or overweight children
- Failure to obtain valid FibroScan results
- Lack of or inadequate histological evaluation

Patients:

We considered 80 unrelated children and adolescents with CHC (diagnosed by positive HCV antibodies and confirmed by detectable serum HCV-RNA by PCR (>50 IU/ml) for over 6 months) from those admitted to the Hepatology Unit of Pediatric Department, Tanta University Hospitals and Pediatric Department of National Liver Institute, Menoufia University, during a period from October 2016 to October 2018. Thirty patients with coexistent causes of other chronic liver disease were excluded, including 5 cases with Wilson disease, 8 cases with autoimmune hepatitis, 6 cases with combined infection with HBV, 8 cases were overweight and obese, 3 cases were below 3 years, whereas 50 patients were included in the study.

Methods:

Patients included in this study were subjected to the following:

A. Complete clinical assessment: with special stress on manifestations of chronic liver disease.

B. Laboratory investigations:

1) Liver function tests: Total and direct serum bilirubin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), gama-glutamyltransferase (GGT), ALP, total serum proteins and albumin, A/G ratio, prothrombin time, activity, and INR [31].
2) Complete blood count (CBC).
3) Renal function tests (Blood urea, serum creatinine).
4) Measurement of fasting blood glucose level: Assessed by using fully automated clinical chemistry auto-analyzer system Konelab 20i (thermo Electron Incorporation, Finland).
5) Measurement of fasting serum insulin level using human insulin enzyme immunoassay test kit according to manufacturer recommendations.
6) Insulin resistance was calculated using the homeostasis model of assessment - insulin resistance (HOMA-IR) [32].
7) Calculations of FIB4 index [33] and Aspartate aminotransaferase to platelet ratio index (APRI) [34].
8) Lipid profile: total cholesterol, high density lipoproteins cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C) and triglycerides (TG) [35, 36].
9) Genotyping assays for *PNPLA3*-I148MGene Variant

C. Liver Stiffness and CAP Measurements Using Transient Elastography (FibroScan).

D. Histological assessment of liver biopsies.

Sample collection

Two samples of blood were taken in the morning after 8 hours fasting. The first was 2.5ml of EDTA anticoagulated blood. It was stored at -20 °C till the time of genotyping assay. The second sample was left to clot at 37 °C then the serum was separated by centrifugation. This sample was used to analyze liver enzymes and lipid profile**.** They were measured by standard chemical and enzymatic commercial methods in the Clinical Pathology Department, Tanta University.

Genotyping assay For PNPLA3-I148M Gene variant by Real-time polymerase chain reaction

Genomic DNA was isolated from EDTA anti-coagulated blood. Sequence variations for *PNPLA3*-I148M was genotyped by a 5′ Nuclease Taqman Assay utilizing the Fast Real-Time PCR instrument [17, 37] by personnel unaware of other patients' data using Pure Link Genomic DNA Mini Kit (Invitrogen, Life Technology-USA) according to Manufacturers' Recommendations.

Genotyping of Adiponutrin variant, Allelic Discrimination plate read and analysis:

The purified DNA was used for adiponutrin gene (PNPLA3 I148M) genotyping. The adiponutrin coding single nucleotide polymorphism (SNP) (rs738409) was genotyped using a solution-phase hybridization reaction and fluorescence detection in the 7300 Real-Time PCR system. PCR reaction contained 20 ng DNA, 900 nM of each primer, 1xTaqMan Universal Master Mix, and 200 nM of VIC-labeled and FAM-labeled probes in 25 ul reaction. Amplification conditions were as follows: 95°C for 10min, 40 cycles of 92°C for 15 s, and 60°C for 1 min. Primer and probe sequences were: forward primer 5´-AACTTCTCTCT CCTTTGCTTTCACA-3´; reverse primer 5´-TCTACAGTGGCC TTATCCCTCC-3´; VIC 5´-TTCCTGCTTCA TGCC-3´; FAM 5´-CCTGCTTCATCCC-3´.

The Real Time PCR software uses the fluorescence values based on the signals from each well. The plotted fluorescence signals indicated which alleles are in each sample.

Liver Stiffness and CAP Measurements Using Transient Elastography (FibroScan)

All patients underwent Fibroscan liver stiffness (LMS) using Transient Elastography (TE). The procedure was performed by an experienced investigator who was blinded to the clinical, laboratory and ultrasound data. Measurements were performed by using the standard technique, as previously described elsewhere [38-40]. According to previous studies on HCV infection the values for different fibrosis stages were as follow: **F0** (0:4.9 kPa), **F1** (5:7 kPa), **F2** (7.1: 9.4 kPa), **F3** (9.5:12.4 kPa) and **F4** (≥12.5 kPa) [39, 41]. Also, as previously published, the CAP score was measured at the exact time and at the same location of LSM and was scored by using the suitable cutoffs into: **No steatosis** (**S0**:< 200 dB/m), **mild steatosis** (**S1**:200-250 dB/m), **moderate *steatosis*** (**S2:** 250-300 dB/m), and **severe steatosis** (**S3**: > 300 dB/m) [42-44].

Histological assessment of liver biopsies:

The minimum biopsy size was 1.7 cm, and the minimum number of portal tracts was 10. Section stained with hematoxylin-eosin and Masson's trichrome and scored for inflammation and fibrosis with the ***METAVIR*** system [45, 46]. One expert pathologist unaware of clinical and genetic data reviewed all biopsies for necro-inflammatory activity grade, fibrosis stage, and steatosis. The ***METAVIR*** scoring system is composed of a two-letter and two-number coding system: **A**= histological activity and **F**= fibrosis. The overall activity scores are defined as follows: **A0**, no activity; **A1**, mild activity; **A2**, moderate activity; **A3**, severe activity. The fibrosis scores are defined as follows: **F0**, no fibrosis; **F1**, portal fibrosis without septa; **F2**, portal fibrosis with few septa; **F3** numerous septa without cirrhosis; F4, cirrhosis [47, 48].

Steatosis is graded as **S0:** absent or <5%; **S1:** 5-33%; **S2:** 34-66%; **S3:** >66% of hepatocytes affected [45, 46].

Statistical analysis

The collected data were organized, tabulated, and statistically analyzed using SPSS software, version 19, SPSS Inc. Chicago, IL, USA). For qualitative data, which describe a categorical set of data by frequency, percentage or proportion of each category, comparison between two groups and more was done using Fisher's Exact Test. For quantitative data, the range, mean, and standard deviation were calculated. For comparison between means of two groups of parametric data of independent samples, student t-test was used. For comparison between means of two groups of non-parametric data of independent samples, Z value of Mann-whitney test was used. For comparison between more than two means of parametric data, F value of ANOVA test was calculated, where scheffe test was performed to compare between each two means if F value was significant. Correlation between variables was evaluated using Pearson's correlation coefficient (r) and spearman rank correlation as indicated. To predict the presence or absence of an outcome based on a set of predictor variables (independent variables) both linear and logistic regression (univariate and multivariate analysis) was done. Logistic regression coefficients (B) are used to estimate Odds ratios (EXP (B) for each of the independent variables. Significance was adopted at p <0.05 for interpretation of results of tests of significance [49, 50].

Results

The general characteristics of the studied patients are shown in **Table (1)**.

Analysis of the PNPLA3 genotypes in the studied patients

The distribution of the PNPLA3 genotype in the studied children with CHC was 34 cases (68.0%) had CC genotype, 9 cases (18%) had CG genotype and 7 cases (14%) had GG genotype **(Table 2).** The number and percentage of each allele is shown in **Table (3)**, where C allele frequency is **77** alleles (**77**%), and G allele frequency is **23** alleles **(23%)** among them.

Table (2): Distribution of PNPLA3 I148M gene variant among the studied patients

Genotype	The studied children with CHC (n=50)	
	N	%
CC	34	68.0
CG	9	18.0
GG	7	14.0

GG: *Homozygous for PNPLA3 I148M gene variant*
CG*: Heterozygous for PNPLA3 I148M gene variant*
CC: *Normal for PNPLA3 gene*

Table (3): Allele frequencies of PNPLA3 gene variants among the studied patients

Allele type	Allele frequencies (n=100) of PNPLA3 gene variant among the 50 studied children with CHC	
	N	%
C	77	**77**
G	23	**23**

G Allele: *Mutant allele*
C Allele: *Normal allele*

Relationship between the genotypes of the studied patients and their demographic, laboratory, FibroScan and histopathological data

Demographic data in relation to the different genotypes

Statistically significant differences were found among the different genotypes of the studied patients as regard the gender, where GG genotype was more frequent among male patients (p=0.0001). However, no significant differences were found among the different genotypes of the studied patients as regard the mean age (p=0.518) **Table (4)**.

Table (4): Genotypes of the studied patients in relation to their demographic data

Demographic data	Genotype of the studied patients (n=50)						χ^2	P
	CC (n=34)		CG (n=9)		GG (n=7)			
	N	%	N	%	N	%		
Sex:								
Female	17	50.0	4	44.4	2	28.6	70.496	**0.0001***
Male	17	50.0	5	55.6	5	71.4		
Age (years):								
Range	7.00-16.00		7.00-15.00		6.00-13.00			
Mean ±SD	10.76±2.65		10.89±2.52		9.57±2.44			
F value	0.668							
P	0.518							

*Statistically significant (P<0.05)

Laboratory results in relation to the different genotypes of the studied patients

Significant higher values of AST, total leucocytic count, blood urea, fasting blood glucose, fasting serum insulin, and HOMA IR, but with lower platelet cell count were found in patients with G allele (CG+ GG) compared to patients without G allele (CC) ($p < 0.05$). While there were no significant differences between patients without G allele (CC) and patients with G allele (CG+ GG) as regard to other parameters including weight, height, BMI, total bilirubin, direct bilirubin, ALT, ALP, GGT, total protein, albumin, prothrombin time, INR, HB, triglycerides, LDL, HDL, total cholesterol, and serum creatinine ($p > 0.05$) **Table (5)**.

Table (5): Clinical and laboratory data of the studied patients according to presence or absence of the G allele

Clinical and laboratory data	The studied children with CHC (n=50)		t-test or Z value	P
	Patients without G allele (CC) (n=34)	Patients with G allele (CG+ GG) (n=16)		
	Mean ±SD	Mean ±SD		
Clinical data:				
Weight (kg)	39.97±10.79	38.50±8.83	0.475	0.637
Height (kg)	142.94±11.70	139.87±11.37	0.872	0.388
Body mass index (BMI) (kg/m^2)	19.20±2.42	19.27±2.04	0.090	0.929
Laboratory data: **Liver function tests:**				
Total bilirubin (mg/dl)	0.78±0.13	0.77±0.11	0.200	0.842
Direct bilirubin (mg/dl)	0.24±0.10	0.23±0.08	0.289	0.774
ALT (U/L)	38.00±9.26	41.00±11.33	0.783	0.437
AST (U/L	40.00±12.06	71.00±19.74	6.898	**0.0001***
ALP (U/L)	166.00±73.08	182.00±104.3	0.633	0.529
GGT (U/L)	37.00±22.37	46.00±21.84	1.393	0.170
Total protein (g/dl)	7.55±0.59	7.36±0.29	1.207	0.233
Albumin (g/dl)	4.05±0.49	4.01±0.44	0.246	0.807
Prothrombin time (seconds)	13.15±0.43	13.12±0.23	0.244	0.809
INR	1.02±0.04	1.02±0.03	0.780	0.439
CBC findings:				
Hemoglobin (HB) (g/dl)	12.76±0.68	12.38±0.90	1.675	0.101
Total leucocytic count (TLC) (x 10^3)	6.58±1.48	7.50±1.42	2.435	**0.015***
Platelet cell count (PLT) (x 10^3)	270.09±39.3	172.31±63.42	4.077	**0.0001***
Lipid profile:				
Triglycerides (mg/dl)	79.23±21.20	87.25±22.48	1.223	0.227
LDL (mg/dl)	75.09±38.43	81.06±43.60	0.491	0.626
HDL (mg/dl)	58.97±22.27	57.56±12.98	0.234	0.816
Total cholesterol (mg/dl)	150.97±36.43	161.69±38.05	0.957	0.343

Blood urea (mg/dl)	29.22±3.62	34.87±13.38	2.313	**0.025***
Serum creatinine (mg/dl)	0.75±0.10	0.80±0.13	1.576	0.122
Fasting blood glucose (mg/dl)	93.03±5.49	107.81±12.35	5.898	**0.0001***
Fasting serum insulin (mU/L)	3.77±0.54	8.32±2.93	8.824	**0.0001***
HOMA IR	0.93±0.46	2.44±1.37	5.793	**0.0001***

*Statistically significant (P<0.05)

FibroScan steatosis CAP values and fibrosis liver stiffness values in relation to the genotypes

The mean values of CAP steatosis by FibroScan among patients with CG and GG genotypes were significantly increased compared to those with CC genotype (P=0.010 and 0.0001 respectively). Also, these values were significantly increased among patients with GG genotype compared to those with CG genotype (P = 0.007). The mean liver stiffness values by FibroScan among patients with CG and GG genotypes were significantly increased compared to those with CC genotype (F =17.713, p = 0.0001) **Table (6).**

Table (6): Steatosis CAP values and fibrosis liver stiffness values by FibroScan among the studied patients in relation to their genotype

FibroScan Parameter	Genotype of the studied patients (n=50)			F value	P	Scheffe test (P)
	CC (n=34)	CG (n=9)	GG (n=7)			
Steatosis CAP values (dB/m)				25.593	**0.0001***	CC vs CG & GG, P=0.010* & 0.0001* CG vs GG, P=0.007*
Range	100-272	280-298	330-390			
Mean ±SD	237.47±53.08	291.44±5.48	366.86±21.96			
Median value	245.00	289.00	370.00			
Liver stiffness values (kPa)				17.713	**0.0001***	CC vs CG & GG, P=0.006* & 0.0001* CG vs GG, P=0.150
Range	1.90-8.90	9.00-9.40	10.00-11.50			
Mean ±SD	7.09±1.97	9.20±0.17	10.87±0.59			
Median value	8.00	9.10	11.00			

*Statistically significant (P <0.05)

Different histopathological parameters in relation to the genotypes

We found that the frequency of cases with higher S2-S3 histopathological steatosis stages among patients with CG and GG genotypes was significantly increased compared to those with CC genotype in whom there was predominance of lower S0-S1-S2 histopathological steatosis stages (χ^2 = 54.011, p = 0.0001). Also, there was a significant increase in the frequency of cases with higher F2- F3 histopathological fibrosis stages among patients with CG and GG genotypes compared to those with CC genotype in whom there is predominance of lower F0-F1-F2 histopathological fibrosis stages (χ^2 = 54.011, p = 0.0001), Furthermore, there was a significant increase in the frequency of cases with higher A2-A3 histopathological activity grades among patients with CG and GG genotypes compared to those with CC genotype in whom there is predominance of lower A1-A2 histopathological activity grades (χ^2 = 52.231, p = 0.0001) **Table (7)**.

Table (7): Frequency of cases with different histopathological steatosis stages, histopathological fibrosis stages and histopathology activity grades among the studied patients in relation to their genotype

	Genotype of the studied patients (n=50)						χ^2 ?	P
	CC (n=34)		CG (n=9)		GG (n=7)			
	N	%	N	%	N	%		

Histopathology steatosis stages								
Stage S0	5	14.7	0	0	0	0	54.011	**0.0001***
Stage S1	5	14.7	0	0	0	0		
Stage S2	24	70.6	9	100	0	0		
Stage S3	0	0	0	0	7	100		
Histopathology fibrosis stages								
Stage F0	5	14.7	0	0	0	0	54.011	**0.0001***
Stage F1	5	14.7	0	0	0	0		
Stage F2	24	70.6	9	100	0	0		
Stage F3	0	0	0	0	7	100		
Histopathology activity grades								
Grade 0	0	0	0	0	0	0	52.231	**0.0001***
Grade 1	19	55.9	0	0	0	0		
Grade 2	15	44.1	8	88.9	0	0		
Grade 3	0	0	1	11.1	7	100		

*Statistically significant (P<0.05)

Multiple logistic regression analysis of predictive factors for histopathological liver steatosis stages, inflammatory activity grades and fibrosis stages among the studied children with CHC

Our results revealed that abnormal AST, CAP value, CG gene variant and GG gene variant could be significant positive predictive factors for histopathological liver steatosis stages among the studied patients ($p < 0.05$), **Table (8).** Also, abnormal AST, abnormal GGT, CAP value, CG gene variant, and GG gene variant could be significant positive predictive factors for histopathological inflammatory activity grades ($p < 0.05$), **Table (9).** In addition, abnormal AST, abnormal GGT, CAP value, liver stiffness value, CG gene variant, and GG gene variant could be significant positive predictive factors for histopathological liver fibrosis stages ($p < 0.05$), **Table (10).**

Table (8): Multiple logistic regression analysis of predictive factors for histopathological liver steatosis stages among the studied patients

Variables	B	SE	P	Exp (B)	95% confidence interval for Exp (B)	
					Lower limit	Upper limit
Male sex	0.847	0.759	0.264	0.429	0.097	1.897
BMI (kg/m2) (>25)	0.082	0.158	0.604	0.921	0.676	1.256
Abnormal ALT	0.212	0.125	0.090	0.809	0.633	1.034
Abnormal AST	0.239	0.107	**0.026***	1.270	1.029	1.567
Abnormal GGT	0.198	0.110	0.071	1.219	0.983	1.511
Abnormal ALP	0.029	0.026	0.255	1.030	0.979	1.083
Abnormal triglycerides	0.004	0.028	0.885	0.996	0.943	1.052
Abnormal HDL	0.012	0.021	0.582	0.989	0.949	1.030
Abnormal LDL	0.028	0.023	0.234	1.028	0.982	1.077
Abnormal total cholesterol	0.043	0.026	0.101	0.958	0.909	1.009
Viral load	0.007	0.005	0.152	0.993	0.984	1.003
HOMA-IR	0.485	0.504	0.337	1.624	0.604	4.364
CAP value (dB/m)	0.110	0.042	**0.009***	1.117	1.028	1.213
CG gene variant	0.301	0.113	**0.028***	1.080	0.995	1.540
GG gene variant	0.118	0.102	**0.034***	1.171	1.128	1.905

B=Logistic Regression Coefficient, SE=Standard Error of B, P=Significance
Exp (B)=Estimated Odds Ratio. *Significant (P<0.05)

Table (9): Multiple logistic regression analysis of predictive factors for histopathological inflammatory activity grades among the studied patients

Variables	B	SE	P	Exp (B)	95% confidence interval for Exp (B)	
					Lower limit	Upper limit
Male sex	0.254	0.587	0.666	0.776	0.245	2.453
BMI (kg/m^2) (>25)	0.145	0.133	0.273	0.865	0.667	1.121
Abnormal ALT	0.068	0.065	0.289	0.934	0.823	1.060
Abnormal AST	0.100	0.044	**0.023***	1.105	1.014	1.204
Abnormal GGT	0.042	0.021	**0.047***	1.043	1.000	1.087
Abnormal ALP	0.004	0.005	0.421	1.004	0.994	1.014
Abnormal triglycerides	0.006	0.021	0.776	1.006	0.965	1.049
Abnormal HDL	0.010	0.020	0.612	1.010	0.972	1.050
Abnormal LDL	0.001	0.019	0.965	1.001	0.964	1.039
Abnormal total cholesterol	0.003	0.019	0.874	0.997	0.960	1.036
Viral load	0.002	0.004	0.643	0.998	0.990	1.007
HOMA-IR	1.115	0.595	0.061	3.050	0.950	9.793
CAP value (dB/m)	0.110	0.042	**0.009***	1.117	1.028	1.213
CG gene variant	0.396	0.162	**0.018***	2.448	0.162	6.012
GG gene variant	0.416	0.155	**0.010***	1.343	0343	5.676

B=Logistic Regression Coefficient, SE=Standard Error of B, P=Significance,
Exp (B)= Estimated Odds Ratio. *Significant (P<0.05)

Table (10): Multiple logistic regression analysis of predictive factors for histopathological liver fibrosis stages among the studied patients

Variables	B	SE	P	Exp (B)	95% confidence interval for Exp (B)	
					Lower limit	Upper limit
Male sex	0.847	0.759	0.264	0.429	0.097	1.897
BMI (kg/m2) (>25)	0.082	0.158	0.604	0.627	0.676	1.256
Abnormal ALT	0.054	0.039	0.169	1.056	0.977	1.140
Abnormal AST	0.112	0.040	**0.005***	1.118	1.034	1.210
Abnormal GGT	0.103	0.041	**0.012***	1.109	1.023	1.202
Abnormal ALP	0.006	0.009	0.467	1.006	0.989	1.024
Abnormal triglycerides	0.006	0.016	0.706	0.994	0.962	1.026
Abnormal HDL	0.016	0.016	0.322	0.984	0.953	1.016
Abnormal LDL	0.006	0.009	0.465	0.994	0.977	1.011
Abnormal total cholesterol	0.015	0.010	0.136	0.985	0.967	1.005
Viral load	0.007	0.005	0.152	0.993	0.984	1.003
HOMA-IR	0.485	0.504	0.337	0.624	0.604	4.364
CAP value (dB/m)	0.004	0.001	**0.001***	1.854	0.712	3.481
Liver stiffness value (kPa)	0.110	0.042	**0.009***	1.117	1.028	1.213
CG gene variant	0.104	0.035	**0.022***	0.625	0.566	0.997
GG gene variant	0.002	0.025	**0.035***	1.016	0.891	1.154

B=Logistic Regression Coefficient, SE=Standard Error of B, P=Significance, Exp (B)=Estimated Odds Ratio.
*Significant (P<0.05)

Discussion

We carried out this study to explore the relationship between PNPLA3-I148M gene variant and the severity of liver steatosis and fibrosis in CHC Egyptian children. In our study **54%** of the studied patients were males and **46%** of them were females. GG genotype was significantly more frequent among male patients and there were no significant differences among the different genotypes of the studied patients as regard the mean age. ***Mackawy et al.*** study in adult Egyptian patients with CHC found that PNPLA3 rs738409 genotype was not

significantly associated with certain age and sex [51]. Also, in line with that finding an earlier study by ***Valenti et al.*** [21].

Progression of chronic HCV disease is slow and a significant proportion of the patients can remain asymptomatic for several years [52]. In our study, the enlarged liver was not clinically palpable, and splenomegaly or signs of portal hypertension were not shown denoting that, no cases had advanced liver disease or cirrhosis among the study population.

In the current study, the distributions of PNPLA3- I148M gene variant in children with CHC were (**68.0%**), (**18%**) and (**14%**) for CC, CG GG genotypes respectively, and the frequency of C allele (the wild Allele) was (**77%**) and that of G allele (the mutant allele) was (**23%**). This prevalence was nearly comparable to that observed in ***Mackawy et al.*** cohort [51]**.** A meta-analysis study by ***Zhang et al.*** showed that individuals with GG genotype had approximately a two-fold higher risk of having CHC when compared to individuals with CC genotype. The GC heterozygous genotype was also associated with a smaller frequency, but still significant [53]. Recently, ***Manchiero et al.*** reported that the prevalence rates of genotypes CC, CG, and GG of the PNPLA3 polymorphism in the included 290 CHC patients, were **45.9%, 21.7%,** and **32.4%**, respectively [54]. In contrast, ***Petta et al.*** reported that a minority (**9.4%**) of CHC patients had the PNPLA3 rs738409 GG polymorphism, compared to **34.8%** and **55.8%** with CG and CC variants, respectively [55].

Hepatic fibrosis may lead to thrombocytopenia as a consequence of impaired synthesis of thrombopoietin**.** Although few data exist on the diagnostic value of platelets count it has been used as a marker of fibrosis and included in many fibrosis scores [56, 57]. In the present work, there was statistically significant lower platelets count in patients with G allele (CG+ GG) compared to patients without G allele (CC). In addition, there was a significant negative correlation between PNPLA3-I148M gene variant and platelets count in the studied patients. In harmony with our findings, was ***Nakaoka et al.*** study in Japanese CHC patients [58]. On the other hand, other studies showed no significant correlation between allele G and platelets count in CHC patients [55, 59].

Plasma liver-enzyme levels are widely used as indicators of liver damage and they are influenced by environmental and genetic factors [60]. In the present work, significantly increased mean AST values were found in patients with CG and GG genotypes compared to those with CC genotype, and there was a significant positive correlation between PNPLA3-I148M gene variant and AST level. In addition, abnormal AST, could be a significant positive predictive factor by logistic regression analysis for histopathological liver steatosis and fibrosis stages as well as inflammatory activity grades. These results could be explained by the suggestion that PNPLA3 rs738409 (G) allele may adversely affect liver functions and it may be involved in the progression of liver fibrosis [17, 51, 58, 61, 62]. Recently, ***Crisan et al.*** reported that AST level was significantly influenced by the adiponutrin polymorphism while no significant differences were found regarding other biochemical liver tests such as ALT or GGT [59].

Metabolic abnormalities associated with overweight/obese patients have been linked to steatosis and fibrosis progression, particularly in nongenotype-3 CHC patients [63, 64]. Therefore, it is to be noted that patients of this study were chosen to have normal weight and height to avoid derangements of metabolic variables present in the overweight/obese patients. In our study, the mean values of lipid profile variables were insignificantly different among the studied children with CHC in relation to their genotypes. Additionally, there were insignificant correlations between PNPLA3-I148M gene variant and all lipid profile variables. These findings are consistent with previously published studies regarding the impact of PNPLA3 rs738409 (G) allele on HCV infected patients [51, 59].

Much attention has focused on the association between HCV infection and glucose intolerance and it was believed that HCV infection could induce IR [65-67]. However, to date the role of the PNPLA3 I148M variant on IR has been controversial [17, 18, 68-73]. In the present work, there were statistically significant higher values of fasting blood glucose, fasting serum insulin, and HOMA-IR in patients with G allele (CG+ GG) compared to patients without G allele (CC). In addition, there were highly significant positive correlations between PNPLA3-I148M gene variant and each of these parameters. A published study by ***Rembeck et al.*** found an association between the PNPLA3 I148M allele and increased IR in CHC in HCV genotype 2, but not genotype 3 infected individuals [68]. Moreover, ***Wang et al.*** reported that I148M allele carriers having higher HOMA-IR levels in normoglycemic subjects from Taiwan [69]. However, many studies failed to find the association of the PNPLA3 I148M variant with IR [17, 18, 70-73]. Recently, ***Crisan et al.*** reported that no significant differences were found regarding HOMA-IR score, blood sugar level, fasting serum insulin among CHC patients according to adiponutrin genotype [59]. Nevertheless, the study showed that the presence of the G allele was a significant predictor for severe steatosis (S2-S3) and patients with severe steatosis had significantly higher blood sugar level. Meanwhile, severe fibrosis was significantly correlated with the presence of *PNPLA3* (G) allele and higher HOMA-IR scores [59].

Previous studies found that PNPLA3 I148M is the most widely replicated genetic variant associated with increased hepatic steatosis [17- 20, 74- 76], and it has been observed that the GG genotype in CHC patients is associated with the greatest risk [21, 22, 24, 54, 62, 77- 80]. In this study, we found that the mean CAP values of CAP steatosis stages among patients with CG and GG genotypes were significantly higher compared to those with CC genotype. In addition, there were significant positive correlations between PNPLA3-I148M gene variant and indicators of hepatic steatosis as assessed by FibroScan with CAP and by histopathology. Furthermore, CG gene and GG gene could be significant positive predictive factors by logistic regression analysis for histopathological liver steatosis stages among the studied children with CHC.

It has been suggested that PNPLA3 rs738409 (G) allele is a reliable predictor for steatosis and fibrosis in CHC and the presence of G allele, along with severe steatosis and IR are significant predictors for fibrosis progression [59]. In the present work, we found a significant increase in the frequency of cases with higher F2- F3 histopathological fibrosis stages among patients with CG and GG genotypes compared to those with CC genotype. Also, the mean LS values by FibroScan among patients with CG and GG genotypes are significantly higher compared to those with CC genotype. In addition, there were significant positive correlations between PNPLA3-I148M gene variant and indicators of hepatic fibrosis as assessed by FibroScan with CAP and by histopathology. Moreover, CG gene variant, and GG gene variant could be significant positive predictive factors by logistic regression analysis for histopathological liver fibrosis stages among the studied patients.

Our findings were in harmony with several studies [21, 37, 54, 62, 81]. ***Fan et al.*** meta-analysis study reported that PNPLA3 rs738409 (C>G) was associated with risk of both advanced liver fibrosis and steatosis in patients with CHC, no matter the infecting HCV genotype. The association was especially strong among Caucasian compared with Asian cohorts although there were no statistically significant differences among them regarding age, BMI, or gender ratio [79]. This result may be related to the IL28B gene polymorphism, which has been confirmed as another factor associated with the severity of liver disease in CHC [82]. Meanwhile, ***Pirazzi et al.*** demonstrated that PNPLA3 is highly expressed in human HSCs suggesting a potential link between HSCs, retinoid metabolism, and PNPLA3 in determining the susceptibility to hepatic fibrosis [83]. Discordant results were shown by ***Huang et al.*** who found that the frequency of the rs738409 G allele was not significantly higher in CHC patients with advanced fibrosis (F3-F4 stages) than in patients with less advanced stages (F0-F2 stages)

[80]. However, this result may have been related to the IFNL3 gene polymorphism, which is another factor associated with the severity of liver disease in CHC as other studies have explained [79, 82].

In the present work, there was a significant increase in the frequency of cases with higher A2-A3 histopathological activity grades among patients with CG and GG genotypes compared to those with CC genotype. Also, CG gene, and GG gene could be significant positive predictive factors by logistic regression analysis for histopathological inflammatory activity grades among the studied patients. Our findings appear to be compatible with that reported by other studies [55, 84]. In contrast, ***Valenti et al.*** found that there was no significant association between rs738409 genotype and Ishak histological necroinflammatory activity [21]. The authors explained that it was difficult to objectively evaluate histological alterations typical of nonalcoholic steatohepatitis (NASH) in the context of HCV induced inflammation, and these hallmarks were not specifically searched for in their study [21].

It has been observed that, compared with patients with the PNPLA3 CC genotype, those with the PNPLA3 CG/GG genotypes had slower biochemical and clinical recovery [85]. Hence, understanding the genetic factors that influence clinical outcomes will help target patients for liver transplant based on individual genetic risk factors and provide insight leading to new therapeutic approaches [85]. Recently published data have shown that silencing PNPLA3 using antisense oligonucleotides might improve features of NAFLD, including fibrosis progression in mice models [86].

Limitations of our study included the small sample size, so we did not have a high proportion of either different genotypes of the PNPLA3 polymorphism or different severities of steatosis and fibrosis in the studied patients. In addition, the interpretation of our findings is limited by the lack of a comparison group (e.g., patients with CLD of other etiologies or healthy volunteers) that could help accurately identify the studied characteristics present in CHC patients. However, despite these limitations, the data obtained could be added to the existing evidence regarding the role of rs738409 polymorphism of the PNPLA3 gene in the severity of hepatic steatosis and advanced fibrosis among children and adolescents with CHC. Moreover, this study examined pediatric patients with CHC, who (to the best of our knowledge) have not yet been studied from these perspectives.

Conclusion

The results of this study showed that polymorphisms in the PNPLA3 I148M gene variant could contribute to the severity of hepatic steatosis and fibrosis in the rs738409 G-allele harboring patients among the studied Egyptian children and adolescents with CHC. Based on the results of this work and other preceding studies, additional prospective studies with a large sample size are warranted for further evaluation of the benefits of PNPLA3 I148M gene variant determination, especially during follow-up of these patients after the recent development of HCV direct-acting antiviral drugs.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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Appendix

Table (1): General characteristics of the studied patients

General characteristics	The studied children with CHC (n=50)	
	Range	**Mean ±SD**
Personal data:		
Age (years)	6.00-16.00	10.62±2.59
Clinical data:		
Weight (kg)	22.00-63.00	39.50±10.14
Weight percentile (%)	25.00-85.00	60.66±18.73
Height (kg)	115.00-165.00	141.96±11.57
Height percentile (%)	5.00-85.00	52.40±24.11
Body mass index (BMI) (kg/m^2)	14.70-23.14	19.22±2.29
Body mass index percentile (%)	10.00-85.00	59.80±17.49
Laboratory data:		
Liver function tests:		
Total bilirubin (mg/dl)	0.57-1.06	0.78±0.12
Direct bilirubin (mg/dl)	0.11-0.40	0.23±0.09
ALT (U/L)	45.00-68.00	39.00±9.91
AST (U/L	52.00-99.00	50.00±20.80
ALP (U/L)	83.00-552.00	171.00±83.58
GGT (U/L)	13.00-100.00	40.00±22.42
Total protein (g/dl)	7.00-9.80	7.49±0.51
Albumin (g/dl)	3.40-5.10	4.04±0.47
A/G ratio	3.56-4.46	3.86±1.86
Prothrombin time (PT) (seconds)	12.00-13.80	13.44±0.37
INR	1.00-1.10	1.02±0.04
CBC findings:		
Hemoglobin (HB) (g/dl)	10.00-14.70	12.64±0.77
Total leucocytic count (TLC) (x 10^3)	3.70-9.60	6.87±1.51
Platelet cell count (PLT) (x 10^3)	100 -380	238.80±66.34
Lipid profile:		
Triglycerides (mg/dl)	40.00-131.00	81.80±21.72
LDL (mg/dl)	38.00-160.00	77.00±39.81
HDL (mg/dl)	35.00-50.00	58.52±19.65
Total cholesterol (mg/dl)	90.00-234.00	154.40±39.91
Blood urea (mg/dl)	23.00-38.00	31.03±8.41
Serum creatinine (mg/dl)	0.50-1.10	0.77±0.11
Fasting blood glucose (mg/dl)	80.00-125.00	97.76±10.75
Fasting serum insulin (mU/L)	3.00-12.00	5.23±2.72
HOMA IR	0.66-6.20	1.41±1.11
APRI	0. 3-3.10	0.43±0.68
FIB4	0.10-1.70	0.44±0.38
PCR HCV (x 10^3)	30.00-350.00	105.34±66.72