15 (2023) 200256

Contents lists available at ScienceDirect

Tumour Virus Research

journal homepage: www.journals.elsevier.com/tumour-virus-research

Association of PNPLA3 (rs738409) & TM6SF2 (rs58542926) and ATG16L1 (rs2241880) genetic variants with susceptibility to hepatocellular carcinoma in a group of Egyptian patients with HCV-induced liver cirrhosis

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1. Introduction

Liver malignancy is considered a global health challenge with increasing incidence. It is expected that, by the year 2025, more than one million individuals will be affected by liver cancer annually [1]. Hepatocellular carcinoma (HCC) represents 90% of liver tumor patients. Hepatitis C virus (HCV) and Hepatitis B virus (HBV) infections represent the main risk factors for HCC, accounting for about 80% of cases [2]. In Egypt, the high incidence of HCC could be attributed to the fact that Egypt has the highest prevalence of HCV worldwide [3]. HCV Patients with liver cirrhosis are one of the high-risk groups for the development of HCC. Non-alcoholic steatohepatitis (NASH), due to diabetes mellitus and metabolic syndrome, is recently considered an important cause of HCC [4]. Many factors affect the prognosis of HCC which include: the status of liver function and tumor staging. Several classifications for HCC staging are present; however, the useful classification system should include size and number of hepatic focal lesions, presence of portal vein invasion and extrahepatic metastasis, serum albumin concentrations, stage of ascites, and portal hypertension [5]. The Barcelona Clinic Liver Cancer staging classification is fulfilling all these criteria. This staging classification is used for the choice of therapeutic options. The prognosis of HCC is very poor because a small percentage of hepatocellular carcinomas can be surgically removed effectively [6]. This fact raises the importance of HCC surveillance among high-risk groups such as liver cirrhosis patients. The recognition of genetic risk factors predisposing to HCC may improve the surveillance programs for HCC. Several genetic studies have proved the exaggerated risk for HCC in patients with alcoholic liver cirrhosis harboring the genetic variants of single nucleotide polymorphisms (SNPs) in the autophagy-related protein 16 like 1 gene, patatin-like phospholipase domain-containing protein 3 gene and transmembrane 6 superfamily gene [7].

Autophagy is one of the main physiological mechanisms that help in the protection of the cells from metabolic toxins [8]. The autophagy catabolic process is dysregulated in many human diseases including cancers [9]. Autophagy has a dual role in carcinogenesis. In the primary stages of neoplasia formation, autophagy plays a tumor-suppressive role to maintain normal homeostasis inside the cells. In later stages of cancer formation, autophagy could induce tumor growth, which is the second role of autophagy in carcinogenesis [10]. The autophagy process is mediated through autophagy-related proteins (ATG). Several studies have shown that the SNPs in ATG genes may be correlated with the risk of many cancers such as melanoma, gastric cancer, colorectal cancer, and HCC [10,11]. The ATG16L1 gene which is located on chromosome 2 plays a vital role in autophagy. One of the most important genetic polymorphisms in the ATG16L1 gene is a SNP located at position 300, which leads to threonine-to-alanine substitution (T300A) in the C-terminus domain of the ATG protein [12]. ATG16L1 (T300A) rs2241880 SNP induces a cleavage site in ATG16L1 protein for caspase-3, which in turn leads to reduction of autophagy, vesicles formation and promotion of inflammatory bowel disease and carcinogenesis [13].

The patatin-like phospholipase domain-containing protein 3 (PNPLA3P) gene is located on chromosome 22 and encodes an enzyme called PNPLA3P [14]. This PNPLA3 enzyme is a membrane lipase located in the hepatocytes and hepatic stellate cells. This explains why the rs738409 (I148 M) in the PNPLA3 gene is considered one of the major genetic risk factors associated with the development of many liver pathologies due to the role of PNPLA3P in the regulation of liver cell fat content [15]. The G allele carriers of the rs738409 c.444C > G SNP in the PNPLA3 gene have a reduced level of the lipase activity of PNPLA3, which furtherly leads to higher hepatic intracellular fat levels, this

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https://doi.org/10.1016/j.tvr.2023.200256
Received 12 November 2022; Received in revised form 16 January 2023; Accepted 10 February 2023
Available online 16 February 2023

dysregulation in the hepatic fat content leads to an elevated risk of liver cirrhosis and HCC [16].

Another SNP associated with impaired intracellular lipid regulation in the hepatic cells is rs58542926, E167K in the transmembrane 6 superfamily member 2 gene, which has a vital role in the development of liver cirrhosis and HCC [17]. TM6SF2 gene is located on chromosome 19, its expression produces the TM6SF2 protein, which is implicated in the risk of NASH and also induces hepatic fibrosis [18]. The T allele of rs58542926 increases the risk of liver disease and HCC development [15]. Research focusing on the relationship between PNPLA3, TM6SF2 and ATG16L1 genetic variants and the risk of HCC was still controversial. Thereby, we performed this study to determine the role of these SNPs with HCC risk in a group of Egyptian patients with HCV-induced liver cirrhosis.

2. Materials and methods

2.1. Ethical approval

Before the start of the study; approval was obtained from the Institutional Review Board of Theodor Bilharz Research Institute (FWA00010609), Informed consent for the study was obtained from all participants. The study was performed following the ethical principles described by the 1964 Declaration of Helsinki and its later amendments.

2.2. Study population

This case-control study was performed from December 2021 to June 2022, on 225 age and sex-matched subjects, divided into three groups:

HCC Group: included 75 patients with HCC on top of HCV infection and liver cirrhosis. The diagnosis had been made by imaging (computed-tomography scan or magnetic resonance imaging), according to the European Association for the study of the Liver (EASL) Clinical Practice Guidelines [19].

Liver cirrhosis Group: included 75 patients with liver cirrhosis due to HCV infection. They were diagnosed by abdominal ultrasound and presence of chronic HCV infection.

Normal control group: included 75 healthy subjects, who were randomly selected from the outpatient clinic. They had normal liver functions and were negative for both HBV and HCV infections.

2.2.1. Inclusion criteria

Adult cirrhotic and HCV-related HCC 18–65 years old patients were recruited from Specialized Medical Hospitals, inpatient departments, and outpatient clinics at Theodor Bilharz Research Institute. Cirrhotic and HCC patients were assessed using the Child-Pugh score.

Normal healthy subjects were subjected to history taking they had normal liver function tests and were serologically negative for both HBsAg & HCV Ab tests, they had no history of diabetes, alcohol abuse or bilharziasis.

2.2.2. Exclusion criteria

- Age below 18 years old.
- Any HCV treatment.
- Presence of HBV or HIV infections.
- Other causes of liver cirrhosis: such as autoimmune hepatitis, and alcoholic & non-alcoholic steatohepatitis.
- Patients who had a history of past malignancies.
- Patients with recurrent or secondary tumors.
- Known drug abuse.
- Patients with spontaneous bacterial peritonitis, or sepsis.
- Any other organ failure: heart failure, respiratory failure, or renal failure.
- Pregnant females.

2.3. Laboratory investigations

* Routine liver and kidney function tests in the form of serum bilirubin level, alanine aminotransferase (ALT) activity, aspartate aminotransaminase (AST) activity, total protein, albumin, alkaline phosphatase (ALP), urea and creatinine were performed for cirrhotic and HCC patients, they were assayed by the Beckman Coulter AU 480 autoanalyzer[1].
- Serum AFP level and hepatitis markers (HBsAg and HCV Ab) were assayed using the chemiluminescence method by kit purchased from Siemens Healthcare Diagnostics[2]
- Complete blood picture (CBC) was performed on Beckman Coulter AcT Diff [1] cell counter.
- Prothrombin concentration (PC) and International Normalized Ratio (INR) were assayed by the automated coagulometer DABE-BEHRING [2].
* Healthy subjects had undergone routine liver & kidney function tests and hepatitis markers.

2.4. Analysis of genetic variants of candidate genes

2.4.1. DNA extraction

Genomic DNA was isolated from the leucocytes using EDTA whole blood samples by the ThermoFisher Scientific [3]GeneJET whole blood genomic DNA Purification Mini Kit. The purified DNA was stored at -20 °C.

The DNA concentration and purity were assayed using Qubit Fluorometric quantification assays supplied by ThermoFisher Scientific[3] and calibrated to 20 ng/μl.[1]

2.4.2. Genotyping and information of candidate SNPs

All participants in the study successfully underwent genotyping for candidate genetic variants of the patatin-like phospholipase domain-containing protein 3, transmembrane 6 superfamily 2 and autophagy-related protein 16 like 1 genes.

Allelic discrimination analysis of genetic variants of the studied genes was performed using real-time PCR TaqMan probes technique according to the protocol proposed by Ref. [20] on the software of step one real-time PCR Applied Biosystems[4]ABI-7500 instrument. The TaqMan minor groove binder probes with non-fluorescent quenchers, they were ready to use supplied by ThermoFisher Scientific *[3]. PNPLA3, rs738409 (Assay ID: C_7241_10), TM6SF2, rs58542926 (Assay ID: C_89463510_10) and ATG16L1, rs2241880 (Assay ID: C_9095577_20).

Programming of thermal cycles of PCR was set, as follows: 95 °C for 10 min, 50 cycles of 92 °C for 10 s and 60 °C for 1 min.[2]

Negative controls were used in PCR runs to exclude any contamination.

* Laboratory tests were done in the Chemical Pathology Department, Theodor Bilharz Research Institute; while molecular analysis of SNPs was done in the Chemical Pathology Department, Cairo University Hospitals.

2.5. Statistical analysis

Data were coded and entered using the statistical package for the Social Sciences (SPSS) version 28 (IBM Corp., Armonk, NY, USA). Based on the Hardy-Weinberg equilibrium, the genotype distributions of the tested SNPs were in a balanced state. Normally distributed quantitative

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[2] Siemens Healthcare Diagnostics Inc.: 511 Benedict Avenue. Tarrytown, NY 10591-5097 USA.
[3] ThermoFisher Scientific, Inc.: 81 Wyman St. Waltham CA. 02451 USA.
[4] Applied Biosystems: Campus (Foster City, California). 850 Lincoln Centre Dr. Foster City, CA. 94404 USA.

variables are summarized using the mean and standard deviation or median and interquartile range for quantitative variables and frequencies (number of cases) whereas categorical variables are expressed as relative frequencies (percentages). Comparisons between groups were done using analysis of variance (ANOVA) with multiple comparisons post hoc test in normally distributed quantitative variables while non-parametric Kruskal-Wallis test and Mann-Whitney test were used for non-normally distributed quantitative variables [21]. For comparing categorical data, the Chi-square ($\chi 2$) test was performed. The exact test was used instead when the expected frequency is less than 5 [22]. Genotype and allele frequencies were compared between groups. Odd's ratio (OR) with 95% confidence intervals (CI) was calculated using binary logistic regression to evaluate the association between SNPs and the study groups (HCC patients, cirrhosis, and control groups). P values less than 0.05 were considered statistically significant throughout the study [23].

3. Results

3.1. Clinical and laboratory data of the study participants

The demographics, clinical, laboratory, and radiological data of cirrhotic, HCC, and normal control groups are presented in Table 1. No statistically significant difference revealed in age and gender composition and bilharziasis among the studied groups. There's a statistical significance difference in encephalopathy grades, CP score, AFP level and liver function tests between HCC and cirrhosis groups.

3.2. Frequency distribution of the Patatin-like phospholipase domain-containing protein 3 (PNPLA3) (C > G) SNP

The frequency distribution of PNPLA3 (rs738409) genotypes, alleles and odd's ratio among the studied groups are represented in Table 2. Table 3 shows PNPLA3 (rs738409) different genotypes in HCC and cirrhosis groups and their relation with the laboratory data.

Results of the association evaluation (table2) revealed that PNPLA3 (rs738409) GG genotype and G allele were significantly increased in HCC patients than in control group [OR = 21.739, 95% CI = 6.011–78.615] and [OR = 5.254, 95% CI = 3.119–8.851] respectively. As well, PNPLA3 (rs738409) GG and CG genotypes were significantly higher in cirrhotic cases when compared to the control group [OR = 41.667, 95% CI = 10.025–173.180] and [OR = 13.352, 95% CI = 5.419–32.903] respectively. In the recessive model, the OR values are doubled meaning that the G allele has an impact on the risk of cirrhosis. Also, the G allele was significantly increased in cirrhotic patients than in the control group [OR = 6.017, 95% CI = 3.566–10.154]. Although the G allele didn't show a statistical significant difference when compared to cirrhotic patients, we found that the CG genotype was significantly more prevalent in cirrhotic patients compared to HCC group [OR = 0.163, 95% CI = 0.063–0.412].

3.3. Frequency distribution of Transmembrane 6 superfamily 2 (TM6SF2) (C > T) SNP

The genotype distributions and allele frequencies of TM6SF2 (rs58542926) polymorphism and odd's ratios among the three studied groups are shown in Table 4. Table 5 compares rs58542926 genetic variants in HCC and cirrhosis groups as regards the laboratory data.

In the analysis of the association between TM6SF2 (rs58542926) polymorphism and HCC risk (Table 4); we found that TT genotype and the T allele were significantly increased in HCC patients than control group [OR = 14.412, 95% CI = 4.708–44.117] and [OR = 5.671, 95% CI = 3.280–9.804] respectively and (P < 0.001). As well, TM6SF2 (rs58542926) genotypes frequency showed significant difference when compared between cirrhotic patients and control group (P < 0.001) as the frequency of TT and CT genotypes were significantly increased in cirrhosis group than control group; [OR = 18.667, 95% CI = 5.537–62.036] and [OR = 9.956, 95% CI = 4.373–22.665] respectively. While the T allele was significantly associated with increased risk for liver cirrhosis when compared to control group [OR = 6.311, 95% CI = 3.649–10.915]. Additionally, we found that the CT genotype was significantly more prevalent in cirrhotic patients compared to HCC group [OR = 0.066, 95% CI = 0.023–0.189]. The median serum creatinine level was significantly lower in TT genotype carriers than in those non-TT genotype carriers (P = 0.029).

3.4. Frequency distribution of autophagy-related protein 16 like 1 (A > G) SNP

Table 6 illustrates the frequency distribution of ATG16L1 (rs2241880) genetic variants, allelic distribution and odd's ratio among

Table 1
Demographic data, clinical, laboratory, and radiological data in the cirrhotic & HCC patients and control groups.

	Control (n = 75)	Cirrhosis (n = 75)	HCC (n = 75)	P
Age (Years)[a]	49.23 ± 6.87	50.15 ± 5.27	50.11 ± 5.51	0.562
Male/Female[b]	60/15	59/16	57/18	0.833
Smoking[c]	0 (0%)	12 (16%)	42 (56%)	**<0.01**
Family history[c]	–	2 (2.7%)	25 (33.3%)	**<0.01**
Bilharziasis[c]	–	23 (30.7%)	28 (37.3%)	0.601
Encephalopathy[c]				
No	–	0 (0.0%)	41 (54.7%)	<
Mild	–	5 (6.7%)	5 (6.7%)	**0.001**
Moderate	–	5 (6.7%)	4 (5.3%)	
Severe	–	65 (86.7%)	25 (33.3%)	
Child Pugh stage[c]				
A	–	44 (58.7%)	29 (38.7%)	**0.023**
B	–	15 (20%)	29 (38.7%)	
C	–	16 (21.3%)	17 (22.7%)	
MELD score[d]	–	11 (7–18)	10 (9–13)	0.678
TLC ($x10^3$ cell/μl)[d]	5.80 (5.0–7.3)	6.50 (5–8.4)	5.60 (4.8–7.0)	0.267
Hb (g/dl)[a]	13.85 ± 1.06a	10.77 ± 2.164b	12.08 ± 1.77c	**<0.001**
Plt($x10^3$/mm3)[d]	265 (198–329) a	130 (93–189) b	157 (128–185)b	**<0.001**
PC (%)[a]	95.12 ± 5.36a	75.45 ± 22.99b	74.49 ± 15.68b	**<0.001**
INR[a]	1.06 ± 0.06a	1.34 ± 0.35b	1.28 ± 0.25b	**<0.001**
Urea (mg/dl)[d]	25 (20–30.5)a	25 (18–63)a	31 (24–41)b	**0.001**
Creatinine (mg/dl)[d]	0.72 (0.62–0.81)a	1 (0.7–1.47)b	0.8 (0.64–1.09)a	**<0.001**
Total protein (g/dl)[a]	7.40 ± 0.56a	6.55 ± 0.98b	7.04 ± 1.29a	**<0.001**
Albumin (g/dl)[a]	4.21 ± 0.34a	3.45 ± 0.9b	3.06 ± 0.66b	**<0.001**
AST (IU/l)[d]	18 (15–25)a	25 (20–73)b	72 (53–98)c	**<0.001**
ALT (IU/l)[d]	16 (10–25)a	29 (16–76)b	34 (19–52)c	**<0.001**
ALP (IU/l)[d]	75 (52–85)a	66 (33–101)a	102 (80–152) b	**<0.001**
TBIL (mg/dl)[d]	0.8 (0.5–0.9)a	0.9 (0.5–2.9)b	1.5 (1–2.4)c	**<0.001**
DBIL (mg/dl)[d]	0.1 (0.1–0.15) a	0.2 (0.1–0.9)b	0.6 (0.3–1)c	**<0.001**
AFP (ng/ml)[d]	–	7.4 (4–28)	68 (15.59–209)	**<0.001**
Liver size[c]				
Average-sized	–	43 (57.3%)	32 (42.7%)	0.072
Enlarged	–	32 (42.7%)	43 (57.3%)	
Spleen status[c]				
Average-sized	–	48 (64%)	24 (32%)	**<0.001**
Enlarged	–	27 (36%)	51 (68%)	

Groups bearing the same initials are not statistically different at P < 0.05.
Groups bearing different initials are significantly different from each other at P = 0.05.
[a] Data are represented as mean ± SD.
[b] Data are represented as a number.
[c] Data are represented as a number (Percent).
[d] Data are represented as median (25th-75th).

Table 2
Frequency distribution of rs738409 SNP genotypic variants and their odd's ratios among the studied groups.

Genotypes	Control (n = 75)	Cirrhosis (n = 75)	HCC (n = 75)	P
CC	50 (66.7%)	8 (10.7%)	23 (30.7%)	**<0.001**
CG	22 (29.3%)	47 (62.7%)	22 (29.3%)	
GG	3 (4%)	20 (26.7%)	30 (40%)	
Alleles	**Control (n = 150)**	**Cirrhosis (n = 150)**	**HCC (n = 150)**	**P**
G allele	28 (18.7%)	87 (58%)	82 (54.7%)	**<0.001**
C allele	122 (81.3%)	63 (42%)	68 (45.3%)	
Genotypes	**HCC (n = 75)**	**Cirrhosis (n = 75)**	**OR (95% CI)**	**P**
GG	30 (40%)	20 (26.7%)	0.522 **(0.195–1.395)**	0.195
CG	22 (29.3%)	47 (62.7%)	0.163 (0.063–0.412)	**<0.001**
CC	23 (30.7%)	8 (10.7%)	Reference	
Alleles	**HCC (n = 150)**	**Cirrhosis (n = 150)**	**OR (95% CI)**	**P**
G allele	82 (54.7%)	87 (58%)	0.873 (0.553–1.379)	0.561
C allele	68 (45.3%)	63 (42%)	Reference	
Genotypes	**Control (n = 75)**	**HCC (n = 75)**	**OR (95% CI)**	**P**
GG	3 (4%)	30 (40%)	21.739 (6.011–78.615)	**<0.001**
CG	22 (29.3%)	22 (29.3%)	2.174 (1.006–4.696)	**0.048**
CC	50 (66.7%)	23 (30.7%)	Reference	
Alleles	**Control (n = 150)**	**HCC (n = 150)**	**OR (95% CI)**	**P**
G allele	28 (18.7%)	82 (54.7%)	5.254 (3.119–8.851)	**<0.001**
C allele	122 (81.3%)	68 (45.3%)	Reference	
Genotypes	**Control (n = 75)**	**Cirrhosis (n = 75)**	**OR (95% CI)**	**P**
GG	3 (4%)	20 (26.7%)	41.667 (10.025–173.180)	**<0.001**
CG	22 (29.3%)	47 (62.7%)	13.352 (5.419–32.903)	**<0.001**
CC	50 (66.7%)	8 (10.7%)	Reference	
Alleles	**Control (n = 150)**	**Cirrhosis (n = 150)**	**OR (95% CI)**	**P**
G allele	28 (18.7%)	87 (58%)	6.017 (3.566–10.154)	**<0.001**
C allele	122 (81.3%)	63 (42%)	Reference	

Data are presented as numbers (percent).
*versus GG.
†versus AA.

the studied groups. While Table 7 compares genotypic variants of rs2241880 in cirrhosis and HCC groups regarding the laboratory data.

To further elucidate the varieties in genotype and allele frequencies between HCV-related HCC and other studied groups regarding ATG16L1 (rs2241880) A > G SNP (Table 6); our findings suggest that GG genotype is significantly associated with higher susceptibility to HCC development among the studied groups. Specifically as the ATG16L1 (rs2241880) GG and AG genotypes were significantly increased in HCC cases than control group [OR = 11.182, 95% CI = 4.549-27.484] and [OR = 4.622, 95% CI = 1.879–11.368] respectively. The frequency of GG genotype was significantly higher in HCC cases than control group [OR = 3.494, 95% CI = 1.326–9.209]. As well the G allele was significantly higher in HCC cases than control and cirrhosis group [OR = 5.113, 95% CI = 3.130–8.354] and [OR = 2.272, 95% CI = 1.415–3.649] respectively.

There was a significant difference in the frequency of ATG16L1 (rs2241880) genotypes when the cirrhotic group was compared to the control group as the GG and AG genotypes were statistically at a higher risk of developing cirrhosis when compared to the control group; [OR = 3.200, 95% CI = 1.265–8.098] and [OR = 6.484, 95% CI = 12.919–14.404] respectively. Also, the G allele was significantly higher in cirrhotic cases than in control group [OR = 2.251, 95% CI = 1.406–3.603]. We found that the median serum urea and creatinine levels were significantly lower in GG genotype carriers (P = 0.04) and (P = 0.02) respectively; while the median AFP level was significantly elevated in GG genotype carriers than in those non-GG genotype carriers (P = 0.019).

Table 3
PNPLA3 (rs738409) genotypes in HCC and cirrhosis groups and the laboratory data.

	GG (n = 135)	CG (n = 15)	CC (n = 15)	P
Hb (g/dl)[a]	12.25 ± 1.65	11.94 ± 1.89	12 ± 1.87	0.793
TLC ($x10^3$ cell/μl)[b]	5.6 (4.5–6.6)	5.25 (3.9–6.5)	6.5 (5.5–7.8)	**0.036**
Plt ($x10^3/mm^3$)[b]	154 (137–189)	157 (128–180)	157 (84–193)	0.785
PC (%)[a]	76.17 ± 14.13	74.68 ± 15.74	72.12 ± 17.82	0.652
INR[a]	1.25 ± 0.21	1.3 ± 0.33	1.29 ± 0.24	0.812
Urea (mg/dl)[b]	30 (24–36.4)	33.1 (24–41)	34.2 (24–53.5)	0.405
Creatinine (mg/dl)[b]	0.8 (0.67–1.04)	0.79 (0.6–1.16)	0.88 (0.72–1.3)	0.351
Total protein (g/dl)[a]	7.11 ± 0.93	7.18 ± 0.98	6.82 ± 1.86	0.612
Albumin (g/dl)[a]	3.05 ± 0.56	3.24 ± 0.72	2.89 ± 0.71	0.210
AST (IU/l)[b]	69 (52–103)	87.5 (54–98)	72 (49–96)	0.716
ALT (IU/l)[b]	34 (20–48)	35.5 (15–65)	33 (20–51)	0.988
ALP (IU/l)[b]	104.5 (84–152)	95 (73–137)	120 (89–157)	0.423
TBIL (mg/dl)[b]	1.3 (1.0–2.4)	1.85 (1.0–2.3)	1.4 (0.9–2.6)	0.733
DBIL (mg/dl)[b]	0.55 (0.3–0.8)	0.55 (0.3–1.1)	0.7 (0.3–1.2)	0.650
AFP (ng/ml)[b]	43.04 (9–155)	56.7 (20.5–154)	100 (36–550)	0.165

[a] Data are represented as mean ± SD.
[b] Data are represented as median (25th-75th).

4. Discussion

In Egypt, HCC is the fourth most common cancer and is one of the most important causes of death [24]. The role of PNPLA3 (rs738409), TM6SF2 (rs58542926) and ATG16L1 (rs2241880) genetic polymorphisms and the risk of liver cancer have gained the attention of many investigators with controversial results. Here in our case-control study, we tried to throw light on the correlation between these clinically relevant SNPs and HCC susceptibility in a group of Egyptian patients who had chronic HCV infection as the cause of HCC development.

Up to our knowledge; the first work recording the prevalence of TM6SF2 (rs58542926) polymorphism in upper African HCV patients was carried out by Ref. [25] who showed that TM6SF2 is not associated with fibrosis or activity progression in Egyptian patients with chronic hepatitis C. While ***Youssef et al., 2021*** revealed that TM6SF2 rs58542926 polymorphism could be associated with the pathogenesis of HCC in chronic HCV patients. These contradictory results encouraged us to investigate the role of these genetic polymorphisms in the pathogenesis of HCC and the possibility of using them as potential non-invasive biomarkers for the development of HCV-related hepatic cirrhosis and progression risk of carcinoma.

The rs738409 in the PNPLA3 gene is one of the genetic factors implicated in the development of several liver pathologies. In patients with HCV infection, this polymorphism was reported to be linked to liver steatosis and progression to fibrosis and cirrhosis but with a less clear association with HCC development with conflicting results [26,27] (Trépo et al., 2016), and [25].

We suggested that PNPLA3 rs738409 (A > G) polymorphism may have an association with the risk of HCC in HCV-related cirrhotic patients, we found that carriers of GG genotype have a 21.7-folds increased risk to develop HCC when compared to non-GG-genotype-carriers, harbors of the G allele are 5 times at more risk to develop HCC than C allele carriers. Also, our findings proved that GG genotype carriers are 41.6 times riskier to progress to liver cirrhosis than those carrying CC genotype while CG genotype carriers are 13 times more prone to this risk and that those harboring the G allele are 6 times more prone to progression to liver cirrhosis than those harboring C allele. Our results are

Table 4
Frequency distribution of TM6SF2 (rs58542926) genetic variants and odd's ratios among the three studied groups.

Genotypes	Control (n = 75)	Cirrhosis (n = 75)	HCC (n = 75)	P
CC	56 (74.7%)	15 (20%)	34 (45.3%)	**<0.001**
CT	15 (20%)	40 (53.3%)	6 (8%)	
TT	4 (5.3%)	20 (26.7%)	35 (46.7%)	
Alleles	**Control (n = 150)**	**Cirrhosis (n = 150)**	**HCC (n = 150)**	**P**
T allele	23 (15.3%)	80 (53.3%)	53.3 (76%)	**<0.001**
C allele	127 (84.7%)	70 (46.7%)	46.7 (74%)	
Genotypes	**HCC (n = 75)**	**Cirrhosis (n = 75)**	**OR (95% CI)**	**P**
TT	35 (46.7%)	20 (26.7%)	0.772 (0.340–1.751)	0.536
CT	6 (8%)	40 (53.3%)	0.066 (0.023–0.189)	**<0.001**
CC	34 (45.3%)	15 (20%)	Reference	
Alleles	**HCC (n = 150)**	**Cirrhosis (n = 150)**	**OR (95% CI)**	**P**
T allele	76 (50.7%)	80 (53.3%)	0.899 (0.571–1.414)	0.644
C allele	74 (49.3%)	70 (46.7%)	Reference	
Genotypes	**Control (n = 75)**	**HCC (n = 75)**	**OR (95% CI)**	**P**
TT	4 (5.3%)	35 (46.7%)	14.412 (4.708–44.117)	**<0.001**
CT	15 (20%)	6 (8%)	0.659 (0.233–1.861)	0.431
CC	56 (74.7%)	23 (30.7%)	Reference	
Alleles	**Control (n = 150)**	**HCC (n = 150)**	**OR (95% CI)**	**P**
T allele	23 (15.3%)	76 (50.7%)	5.671 (3.280–9.804)	**<0.001**
C allele	127 (84.7%)	74 (49.3%)	Reference	
Genotypes	**Control (n = 75)**	**Cirrhosis (n = 75)**	**OR (95% CI)**	**P**
TT	4 (5.3%)	20 (26.7%)	18.667 (5.537–62.036)	**<0.001**
CT	15 (20%)	40 (53.3%)	9.956 (4.373–22.665)	**<0.001**
CC	56 (74.7%)	15 (20%)	Reference	
Alleles	**Control (n = 150)**	**Cirrhosis (n = 150)**	**OR (95% CI)**	**P**
T allele	23 (15.3%)	80 (53.3%)	6.311 (3.649–10.915)	**<0.001**
C allele	127 (84.7%)	70 (46.7%)	Reference	

Data are presented as numbers (percentage).
*versus GG.
†versus AA.

Table 5
Comparison between TM6SF2 (rs58542926) genetic variants in HCC and cirrhosis groups as regarding the laboratory data.

	TT (n = 135)	CT (n = 15)	CC	P
Hb (g/dl)[a]	12.21 ± 1.45	13.18 ± 1.50	11.76 ± 2.05	0.163
TLC (x10^3 cell/μl)[b]	5.5 (4.2–6)	6.35 (5.2–6.6)	6.3 (5.1–7.3)	0.121
Plt (x10^3/mm^3)[b]	159 (137–200)	141.5 (83–157)	152 (110–180)	0.278
PC (%)[a]	75.05 ± 12.97	73.93 ± 16.04	74.02 ± 18.39	0.961
INR[a]	1.27 ± 0.24	1.25 ± 0.16	1.29 ± 0.29	0.925
Urea (mg/dl)[b]	28 (24–40.7)	25 (21–38)	34.2 (30–53.5)	0.069
Creatinine (mg/dl)[b]	0.80 (0.60–0.88)	0.86 (0.80–0.98)	0.91 (0.71–1.30)	**0.029**
Total protein (g/dl)[a]	7.17 ± 0.94	7.6 ± 0.9	6.81 ± 1.61	0.279
Albumin (g/dl)[a]	3.14 ± 0.59	2.82 ± 0.62	3.01 ± 0.73	0.464
AST (IU/l)[b]	75 (53–102)	88.5 (71–98)	68 (49–93)	0.528
ALT (IU/l)[b]	34 (20–60)	51.5 (48–58)	29.5 (18–43)	0.141
ALP (IU/l)[b]	106 (87–152)	144.5 (120–150)	98.5 (73–157)	0.389
TBIL (mg/dl)[b]	1.5 (1–2.3)	1.94 (1.2–2.5)	1.4 (0.9–2.8)	0.869
DBIL (mg/dl)[b]	0.5 (0.3–0.9)	0.76 (0.7–1.2)	0.5 (0.2–1.1)	0.497
AFP (ng/ml)[b]	34.6 (12.5–154)	70 (7.5–108)	93.1 (20.5–380)	0.103

[a] Data are represented as mean ± SD.
[b] Data are represented as median (25th-75th).

supported by the results reported by Ref. [28] and came following that of [29] who showed that the percentage of hepatic cirrhosis patients was significantly elevated among the GG genotype patients than CC/GC patients (P = 0.026). Contrary to our results [30,31], who made a study on American, European and Japanese ethnic groups respectively proved that PNPLA3 is not a significant risk factor for HCC among patients with HCV.

We found that TT genotype and T allele of TM6SF2 (rs58542926) had a significant association with HCC development which elucidate the functional effect of this polymorphism in HCV-induced HCC Egyptian patients; finding that those carrying TT genotype are 14 times riskier of developing HCC and that those carrying the T allele are 5 times at more risk to develop HCC than those with C allele. As well our findings elaborated that TT genotype carriers are 18 times riskier to progress to cirrhosis than those carrying CC genotype while CT genotype carriers are 9.9 times more prone to this risk and that those harboring T allele are 6 times more prone to progression to liver cirrhosis than those harboring C allele.

Results of our study come following the reports from Ref. [32] on European Caucasian populations who revealed that the T allele of the TM6SF2 gene might have an impact on HCC development in fatty liver disease patients which. Also, our results go hand in hand with results from a meta-analysis [17] which showed that the risk of HCC in the TT genotype carriers was significantly increased than in other genotypes groups.

As [33] mentioned that PNPLA3 rs738409 genotypic and allelic distributions show an ethnic difference in its frequency, we expected that studies on Arabic ethnic patients will show the same results as ours, which is supported by findings of [34] who showed that PNPLA3 GG genotype carriers had an exaggerated risk of HCC occurrence and that GG genotype carriers had a 3-folds elevated risk when compared to non-GG genotype carriers in Moroccan patients with chronic hepatitis C. In contradictory to our results [28]; showed that TM6SF2 rs58542926 is associated with HCC development in alcohol-related cirrhosis patients, but not with HCC development in chronic HCV cirrhotic patients. Also [35] assessed the interaction between PNPLA3 rs738409 and TM6SF2 rs58542926 variants in the conditioning of HCC development; their study was conducted on 511 cirrhotic patients (44% alcohol-related and 56% viral-related) who were retrospectively investigated for HCC occurrence, finding that patients with HCC were more likely to be PNPLA3 GG homozygotes (41/150 vs. 60/361, p = 0.009) and TM6SF2 CT or TT (27/150 vs. 41/361, p = 0.044); concluding that TM6SF2 C/T or T/T in conjunction with PNPLA3 G/G variants may be potential genetic risk factors for developing HCC in alcohol-related cirrhosis (p = 0.0007) but not in viral cirrhosis. This difference may be attributed to different ethnicity in addition to different HCV genotypes. Further studies are needed to assess the correlation between these SNPs and post-HCV HCC in different ethnic populations and various HCV genotypes to clarify this correlation.

It becomes clear that in studying the causal effect of the PNPLA3 rs738409 [G] allele and TM6SF2 rs58542926 [T] allele on the risk of HCC, the severity of the underlying liver disease must be taken into account which is supported by Ref. [36] who demonstrated that G allele carriers of PNPLA3 rs738409 with HCV cirrhosis of lower viral load, develop liver failure at a younger age and may have a real impact on the timing and need of liver transplantation for chronic liver fibrosis in both allelic and recessive models (CG + GG vs. CC: OR = 1.90; 95% CI = 1.017–3.472, P = 0.045 and GG vs. CC + CG: OR = 2.94; 95% CI = 1.032–7.513, P = 0.042). But our study couldn't show a significant difference regarding the correlation of these SNPs with tumor characteristics and prognosis of HCC in the studied patients, this is in line with

Table 6
Frequency of ATG16L1 (rs2241880) genetic variants & allelic distribution and odd's ratios among the studied groups.

Genotypes	Control (n = 75)	Cirrhosis (n = 75)	HCC (n = 75)	P
AA	42 (56%)	15 (20%)	11 (14.7%)	<
AG	19 (25.3%)	44 (58.7%)	23 (30.7%)	**0.001**
GG	14 (18.7%)	16 (21.3%)	41 (54.7%)	
Alleles	**Control (n = 150)**	**Cirrhosis (n = 150)**	**HCC (n = 150)**	**P**
A allele	103 (68.7%)	74 (49.3%)	45 (30%)	<
G allele	47 (31.3%)	76 (50.7%)	105 (70%)	**0.001**
Genotypes	**HCC (n = 75)**	**Cirrhosis (n = 75)**	**OR (95% CI)**	**P**
GG	41 (54.7%)	16 (21.3%)	3.494 (1.326–9.209)	**0.011**
AG	23 (30.7%)	44 (58.7%)	0.713 (0.282–1.802)	0.474
AA	11 (14.7%)	15 (20%)	**Reference**	
Alleles	**HCC (n = 150)**	**Cirrhosis (n = 150)**	**OR (95% CI)**	**P**
G allele	105 (70%)	76 (50.7%)	2.272 (1.415–3.649)	**<0.001**
A allele	45 (30%)	74 (49.3%)	**Reference**	
Genotypes	**Control (n = 75)**	**HCC (n = 75)**	**OR (95% CI)**	**P**
GG	14 (18.7%)	41 (54.7%)	11.182 (4.549–27.484)	**<0.001**
AG	19 (25.3%)	23 (30.7%)	4.622 (1.879–11.368)	**<0.001**
AA	42 (56%)	11 (14.7%)	**Reference**	
Alleles	**Control (n = 150)**	**HCC (n = 150)**	**OR (95% CI)**	**P**
G allele	47 (31.3%)	105 (70%)	5.113 (3.130–8.354)	**<0.001**
A allele	103 (68.7%)	45 (30%)	**Reference**	
Genotypes	**Control (n = 75)**	**Cirrhosis (n = 75)**	**OR (95% CI)**	**P**
GG	14 (18.7%)	16 (21.3%)	3.200 (1.265–8.098)	**0.014**
AG	19 (25.3%)	44 (58.7%)	6.484 (12.919–14.404)	**<0.001**
AA	42 (56%)	15 (20%)	**Reference**	
Alleles	**Control (n = 150)**	**Cirrhosis (n = 150)**	**OR (95% CI)**	**P**
G allele	47 (31.3%)	76 (50.7%)	2.251 (1.406–3.603)	**<0.001**
A allele	103 (68.7%)	74 (49.3%)	Reference	

Data are presented as numbers (percentage).
*versus GG.
†versus AA.

Table 7
Comparison between ATG16L1 (rs2241880) genotypic variants in HCC and cirrhosis groups regarding the laboratory data.

	GG (n = 135)	AG (n = 15)	AA (n = 15)	P
Hb (g/dl)[a]	12.31 ± 1.67	12.14 ± 1.61	11.12 ± 2.25	0.138
TLC (x10^3 cell/μl)[b]	5.5 (4.5–6.6)	5.6 (4.8–7.0)	6.5 (5.6–8.0)	0.339
Plt (x10^3/mm^3)[b]	164 (140–189)	140 (110–170)	146 (77–204)	0.126
PC (%)[a]	74.71 ± 15.19	75.57 ± 13.46	71.44 ± 22.02	0.771
INR[a]	1.27 ± 0.23	1.25 ± 0.22	1.36 ± 0.4	0.478
Urea (mg/dl)[b]	30 (23.5–36.4)	31 (26–41)	41 (32.1–79.2)	**0.040**
Creatinine (mg/dl)[b]	0.80 (0.60–0.91)	0.83 (0.77–1.05)	1.04 (0.81–1.50)	**0.020**
Total protein (g/dl)[a]	7.36 ± 0.87	6.73 ± 1.66	6.49 ± 1.52	0.053
Albumin (g/dl)[a]	3.04 ± 0.61	3.19 ± 0.67	2.82 ± 0.81	0.306
AST (IU/l)[b]	79 (57–98)	62 (53–98)	79 (49–96)	0.446
ALT (IU/l)[b]	34 (19–49)	34 (20–54)	37 (17–55)	0.904
ALP (IU/l)[b]	103 (81–148)	97 (79–181)	133 (99–183)	0.472
TBIL (mg/dl)[b]	1.5 (0.9–2.4)	1.6 (1.2–2.2)	1.0 (0.9–5.6)	0.911
DBIL (mg/dl)[b]	0.6 (0.3–1.0)	0.6 (0.4–0.9)	0.5 (0.2–3.3)	0.858
AFP (ng/ml)[b]	200 (13.6–2505)	100 (34.6–249)	36 (9–106)	**0.019**

[a] Data are represented as mean ± SD.
[b] Data are represented as median (25th-75th).

the results of [37].

Dysregulation of genetic factors affecting hepatic fat accumulation will lead to the promotion of HCC pathogenesis and could be fruitful biomarkers for patients' categorization [38]. showed that the genetic variants of PNPLA3 and TM6SF2 were strongly associated with HCC, with an exaggerated risk in NAFLD patients harboring five risk alleles as compared to non-carriers of these 5 alleles [39]. have evaluated a multi-gene score including the genetic variants of PNPLA3, TM6SF2, and HSD17B13 genes and found that patients with these variants are at increased risk for cirrhosis and HCC development as compared to the general population. All these studies throw light on the possibility of using these genetic scores to expect the development of liver disease in metabolic syndrome patients which helps in the choice of therapeutic options [40].

Loss of the autophagy related protein (ATG16L1) is related to inflammation and tumor formation. ATG16L1 polymorphisms relation with cancer has been confirmed in many cancers such as thyroid cancer [41], gastric cancer [42,43], oral squamous cell carcinoma [44] colorectal cancer [45] and lung cancer [46]. Our study shows that ATG16L1 polymorphism is a potential risk factor for HCC development in HCV-related cirrhotic patients. We illustrated an overall significant association between the ATG16L1 (rs2241880) and increased risk for HCC; finding that those carrying GG/AG genotypes are 11/4.6 times more risky to develop HCC than those with CC genotype and that those harboring the G allele are 5 times riskier to develop HCC than those with C allele. Moreover, our findings proved that GG genotype carriers are 3.2 times riskier to progress to cirrhosis than those carrying the AA genotype while AG genotype carriers are 6.4 times more prone to this risk and that those harboring the G allele are 2 times more prone to progression to liver cirrhosis than individuals with C allele. This is concordant with [47] study which reported that the ATG16L1 G allele was more prevalent in HCC patients when compared to controls (P = 0.022). G allele carriers were 1.76 folds at more risk to develop HCC (ORs = 1.76, 95% CI: 1.07–2.88).

5. Conclusions

Our findings indicate that PNPLA3, TM6SF2 and ATG16L1 genetic variants may contribute in the development of HCC in post-HCV cirrhosis Egyptian patients; suggesting that these genetic polymorphisms play potential roles in the process of liver carcinogenesis and raising the importance to consider using them as biomarkers in HCC high-risk groups such as chronic HCV cirrhotic patients which will help the surveillance for HCC and early detection of HCC which will improve the outcome. Also, this will help in HCC risk evaluation in chronic liver disease patients.

Funding

This work was self-financed, we did not receive any specific grant from funding agencies in the public, commercial or not-for-profit sectors.

Declaration of competing interest

All authors have no actual or potential conflicts of interest.

Data availability

The data that has been used is confidential.

Acknowledgment

This work was approved and supported by Theodor Bilharz Research Institute.

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