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The impact of polymorphism in PNPLA3 and TM6SF2 genes on the susceptibility and survival of hepatitis C-related hepatocellular carcinoma

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Abstract

Background: Genetic variants of Patatin-like phospholipase domain-containing protein 3 (PNPLA3) and transmembrane 6 superfamily member 2 (TM6SF2) genes have been reported with the development of hepatocellular carcinoma (HCC). This study aims to explore the role of The PNPLA3 rs738409 and TM6SF2 rs58542926 single-nucleotide polymorphisms (SNPs) on the incidence and survival of HCV-induced HCC in Egyptians.

Methods and results: This case-control study included (120) HCC and (144) hepatitis C virus (HCV) patients. Baseline clinical, laboratory, tumor characteristics data, HCC recurrence, and overall survival were collected. PNPLA3 rs738409 and TM6SF2 rs58542926 polymorphism were detected by TaqMan allelic discrimination assay. We found that HCC patients were significantly older with male predominance. A significant difference between the TT genotypes of TM6SF2 frequency was observed in HCC compared with HCV patients. Moreover, the T allele of TM6SF2 distributions revealed a significant contribution to the different stages of HCC (p=0.03). Both PNPLA3 rs738409 and TM6SF2 rs58542926 variants showed a significant relation with treatment response according to the modified RECIST criteria. Age and diabetes mellitus were the independent factors associated with the development of HCC by multivariate regression analysis.

Conclusions: TM6SF2 rs58542926 polymorphism, not PNPLA3 rs738409, could be implicated in the development of HCV-induced HCC and its progression.

Keywords: Hepatocellular carcinoma (HCC), PNPLA3, TM6SF2, Single-nucleotide polymorphism, Chronic hepatitis C

Introduction

Hepatocellular carcinoma (HCC) classifies the sixth most common type of malignancy worldwide [1]. The prevalence of HCC diverges by geographic region according to its epidemiological data. In Egypt, liver cancer is ranked 3rd and 15th in Africa and worldwide, respectively, and is the most common cause of mortality and morbidity-related cancer [2].

Environment-related risk factors such as both hepatitis B virus (HBV) and hepatitis C virus (HCV) and other predisposing factors including non-alcoholic fatty liver disease (NAFLD), diabetes, obesity, and smoking are associated with increased HCC risk by approximately 20 fold [3, 4]. It was also confirmed that genetic mutations affect the susceptibility to liver cancer [5]. Genetic factors are related to the pathogenesis of liver cancer, and these

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factors increase the differences between individuals in the susceptibility to diseases [5, 6].

Patatin-like phospholipase domain-containing protein 3 (PNPLA3), adiponutrin, is a multifunction enzyme encoded by PNPLA3gene and is located on chromosome 22 [7]. It is highly expressed in the liver and adipose tissue, and also, it contributes to carbohydrate and lipid metabolism in the liver [8].

Recently, several studies have revealed that there is an association between altered PNPLA3 expression and multiple chronic liver diseases such as alcoholic liver disease and non-alcoholic fatty liver disease [9, 10].

In genome-wide association (GWA) studies, the PNPLA3 SNP, rs738409 C >G (Ile148Met), was reported in humans. This variant was associated with the development of NAFLD [11, 12]. Recent studies have demonstrated an important role of rs738409 SNP in liver cancer risk [13, 14]. Patients carrying mutant homozygote G allele had elevated hepatic triglyceride levels and increased serum ALT levels [15]. Also, PNPLA3 (rs738409: C> G) may affect the severity of fibrosis in patients with fatty liver [16, 17]. These last findings could stimulate hepatocarcinogenesis through dysregulation in lipid metabolism and inflammatory mediators [18].

Another important gene polymorphism that has a significant role in lipid metabolism and chronic liver disease is the transmembrane 6 superfamily member 2 (TM6SF2) gene which is located on chromosome 19. A study revealed that a significant association was found between TM6SF2 rs58542926 and NAFLD [12]. Also, Musso et al. [19] have shown that the TM6SF2 variant had a significant effect on nutrient oxidation, glucose, and lipid metabolism in NAFLD patients. Another study in obese children found that TM6SF2 (c.499A> G) was a significant association with lower levels of total cholesterol and low-density lipoprotein cholesterol, indicating that it could enhance liver injury [20].

This study aimed to investigate the relationship between the PNPLA3 rs738409 and TM6SF2 rs58542926 polymorphisms and hepatitis C-induced HCC occurrence, recurrence, and survival.

Subjects and methods

Subjects

A total of 264 subjects were recruited in this study including patients (120) who had HCC and (144) patients infected with chronic hepatitis C (CHC) genotype 4. HCC patients were recruited from the multidisciplinary HCC Clinic, Kasr Alainy Hospital, Cairo University, Egypt, while HCV patients were enrolled at the National Hepatology and Tropical Medicine Research Institute (NHTMRI), Cairo, Egypt.

HCC was diagnosed according to the criteria in the guidelines of the American Association for the study of Liver Diseases (AASLD), using computerized tomography (CT) or magnetic resonance imaging (MRI) techniques and alpha-fetoprotein (AFP) [21]. Inclusion criteria for all patients were (i) lack of co-infection with HBV, HIV, EBV, and CMV; (ii) no history of alcohol consumption; (iii) no bilharzias; and (iv) no suffering from other autoimmune or hematological diseases. HCC patients were treatment naïve.

Patients were subjected to the following: Full history taking and clinical assessment. Baseline laboratory tests were collected in the form of complete blood count, liver function tests, renal functions, and alpha-fetoprotein (AFP) measurements in addition to tumor characteristics (focal lesion site, size and number, portal vein, and abdominal lymph node assessment). HCV infection was diagnosed using a quantitative real-time polymerase chain reaction (PCR) for HCV RNA (Cobas Amplicor, HCV Roche, Branchburg, NJ, USA, v 2.0, detection limit 15 IU/mL).

HCC patients were assessed by the Eastern Cooperative Oncology Group performance status (PS) [22] and managed according to the Barcelona Clinic Liver Cancer (BCLC) guideline [23]. Response to treatment was rated using the modified Response Evaluation Criteria in Solid Tumors (mRECIST) guidelines [24].

Follow-up: The initial evaluation of HCC treatment response was done after 1 month by triphasic CT or MRI then every 3 months for 2 years and then return to routine surveillance every 6 months. Follow-up was performed till patients' death or till the end of the study [25].

Blood sample and DNA isolation

A 5-ml blood sample was collected from each individual in sterile anticoagulant tubes. The extraction and purification of genomic DNA from the peripheral blood lymphocytes were conducted using a QIAamp DNA Mini and Blood Mini kit (Qiagen #51104) according to the manufacturer's instructions and preserved at −80 °C for genetic determinations [26]. Briefly, add 200μl of the whole blood and 20μl of Qiagen protease into a 1.5-ml micro-centrifuge tube, then add 200μl of buffer (AL) and mix by pulse vortexing for 15 s. This was followed by incubation in a water bath of 56 °C for 10 min. Add 200μl of ethanol (100%) to the sample and mix again by pulse vortexing for 15 s. Then, transfer carefully into a QIAamp mini spin column (in a 2 ml collection tube), centrifuge the tube at 8000rpm for 1 min, place the QIAamp mini spin column in a clean 2-ml collection tube, discard the tube containing the filtrate, add 500μl of buffer (AW1), and centrifuge at 8000 rpm for 1 min. Place the QIAamp mini spin column in a clean 2-ml collection tube, add

500µl of buffer (AW2), centrifuge at 14,000 rpm for 3 min, place the spin column in a new 2-ml collection tube, and centrifuge at 14,000rpm for 1 min. Place the QIAamp mini spin column in a clean 1.5-ml micro-centrifuge tube, add 200µl of elution buffer (AE), and incubate at room temperature for 1 min. Lastly, centrifuge the mixture at 8000rpm for 1 min, discard the QIAamp mini spin column, and store the micro-centrifuge tube containing the eluted DNA at −80 °C (ref: Qiagen 2017). QIAamp DNA mini kit (Qiagen) was used with the extraction of DNA from the blood samples [27].

Genotyping of PNPLA3 rs738409 and TMS6F2 rs58542926 SNPs

After DNA extraction, the samples of all patients were subjected to the real-time PCR reaction to analyze the polymorphism of the two genes and the initial step was to bring the concentration of DNA of each sample to 20 ng/µl. So, samples were diluted to reach this value. Then, genotyping of PNPLA3 rs738409 and TMS6F2 rs58542926 was performed for all patients by real-time PCR and using the system "Taqman allelic discrimination assay" on Agilent Mx3000p qPCR, real-time PCR (Agilent Technologies, Germany). The assay was standardized in a final volume of 25 µl: 12.5 µl of 2× TaqMan Universal MasterMix II, no UNG (Applied Biosystems, USA), 1.25 µl of Genotyping Assay 20×, 10.25 µl of Dnase-free water (Promega, USA), and 1 µl of genomic DNA. The cycling was as follows: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The interpretation of genotypes for PNPLA3 rs738409 and TMS6F2 rs58542926 was given by (CC, CG, GG, and CC, CT, and TT, respectively) [26]. At the end of PCR amplification, the endpoint plate reading was analyzed using the software of Agilent Mx3000p qPCR which uses the fluorescence measurements made during the plate read to plot fluorescence (Rn) values based on the signals from each well) [26].

Statistical analysis

All statistical analyses were performed using the SPSS program for Windows (version 20 statistical software; Texas instruments, IL, USA). Categorical variables are given as the number and percentage. Continuous data are expressed as the mean and standard deviation or as median with the interquartile range (25–75%). A comparison between distributions of categorical variables was performed using chi-square (X^2) test. In addition, variables were described as odds ratio (OR) with a 95% confidence interval (95% CI) where appropriate. The data were considered significant if the p value was < 0.05 and highly significant if p < 0.01. The associations between the gene polymorphisms and HCC stages were tested using the crosstabs test. Kaplan-Meier method was used to calculate the survival rates, and the log-rank test was used to test the significance of the difference in the patients' survival [28].

Biostatistical study design

We are planning a study of subjects in which we will regress the values of the patients against the studied techniques. Prior data indicate that the standard deviation of the control is 0.5, and the standard deviation of the regression errors will be 1.7. If the true slope of the line obtained by regressing patients against control is 2.3, we will need to study 120 subjects for each group to be able to reject the null hypothesis that this slope equals zero with a probability (power) of 95%. The type I error probability associated with this test of this null hypothesis is 0.05.

Results

Characteristics of the studied patients

A total of 264 subjects were analyzed in our study, including 120 patients with HCC and 144 patients with hepatitis C virus (HCV) but without HCC. The characteristics of all the patients are described in (Table 1). Patients with HCC (n = 120) were significantly older with male predominance. They had significantly higher serum total bilirubin, AFP and lower hemoglobin, platelet count, serum albumin, and alanine aminotransaminase (ALT).

Association of PNPLA3 rs738409 and TMS6F2 rs58542926 variants with HCC

We examined if the frequencies of SNPs were associated with the HCC development in patients with HCV-related HCC (Table 2). Our data showed that the frequencies of PNPLA3 GG, CG, and GG genotypes did not differ significantly between HCV and HCC patients. On the contrary, the frequencies of the TM6SF2 TT genotype were significantly higher in HCC compared with HCV patients (Table 2), indicating a role of this genotype in HCC development.

Association of PNPLA3 rs738409 and TMS6F2 rs58542926 variant with HCC characteristics, staging, and response to treatment

We investigated the association between polymorphisms at PNPLA3 rs738409, TM6SF2 rs58542926, and HCC clinical characteristics (Table 3), but there was no significant correlation between the two polymorphisms and any of the patients and tumor characteristics. On the contrary, we observed a significant (P=0.03) association between TM6SF2 rs58542926 polymorphism and disease stage with a higher frequency of TM6SF2 T allele in late-stage HCC patients (0.313%) compared to early and

Table 1 Demographic and clinical characteristics of HCV and HCC patients

			HCV *N*=144	HCC *N*=120	*P* value
Demographic data	**Age (years)**		46.8±9.8	61.5±7.2	0.001**
	Gender	**Female**	71 (49.3%)	30 (25.0%)	0.001**
		Male	73 (50.7%)	90 (75.0%)	
	Smoker		14 (9.7%)	34 (28.3%)	0.001**
	Diabetes		26 (18.1%)	33 (27.5%)	0.04*
	BMI		28.7±4.0	27.8±5.4	0.1
Laboratory investigation	**Hb**		13.5±1.6	12.9±2.0	0.01*
	WBC		5.9±1.9	6.7±2.8	0.2
	Platelets		180.1±65.7	159.4±71.9	0.01*
	INR		1.0±0.1	1.2±0.3	0.001**
	ALT		60.0 (41.0–86.0)	50.0 (31.0–75.8)	0.01*
	AST		61.0 (43.0–87.0)	58.5 (35.0–80.5)	0.2
	Alb		3.9±0.6	3.6±0.6	0. 01*
	Bil.T		0.8 (0.6–1.0)	0.96 (0.7–1.4)	0.001**
	AFP		6.0 (2.8–12.6)	61.2 (9.6–242.1)	0.001**
	Crea.		0.9±0.5	0.9±0.2	0.5
HCV F. grades	**F1**		43 (29.9%)	29 (24.2%)	0.3
	F2		27 (18.8%)	7 (5.8%)	0.01*
	F3		42 (29.2%)	11 (9.2%)	0.001**
	F4		32 (22.2%)	73 (60.8%)	0.001**
BCLC stages	**Early**		-	56 (46.7%)	-
	Intermediate		-	44 (36.7%)	-
	Late		-	16 (13.3%)	-
	Advanced		-	4 (3.3%)	-

Age, body mass index (BMI), hemoglobin (Hb), white blood cells (WBC), platelets, international normalized ratio (INR), albumin (Alb), and creatinine (Crea) are represented as the mean ± SD; the data were analyzed by Student's *t* test. While ALT, AST, total bilirubin (Bil.T), and AFP are represented as the median with interquartile range (25–75%), the data were analyzed by Mann-Whitney *U* test and gender, smoker, DM, and HCV F. grades/BCLC stages are represented as frequency and percent; the data were analyzed by X^2 test

* *P* value ≤ 0.05 significant; ** *P* value ≤ 0.01 highly significant

Table 2 Genotype distribution of PNPLA3 rs738409 and TM6SF2 rs58542926 in HCV and HCC patients

		HCV *N*=144	HCC *N*=120	[a]*P* value	OR	95% C.I	[b]*P* value
PNPLA3 rs738409	**CC**	72 (50.0%)	57 (47.5%)	0.6	1 (reference)		
	CG	58 (40.3%)	48 (40.0%)	0.9	1.045	0.624–1.753	0.8
	GG	14 (9.7%)	15 (12.5%)	0.4	1.353	0.604–3.033	0.4
	C Allele	202 (0.701)	162 (0.675)	0.6	1 (reference)		
	G Allele	86 (0.299)	78 (0.325)		1.131	0.781–1.637	0.5
TM6SF2 rs58542926	**CC**	107 (74.3%)	84 (70.0%)	0.3	1 (reference)		
	CT	35 (24.3%)	28 (23.3%)	0.8	1.019	0.574–1.808	0.9
	TT	2 (1.4%)	8 (6.7%)	0.03*	5.095	1.054–24.629	0.04*
	C Allele	249 (0.865)	196 (0.817)	0.2	1 (reference)		
	T Allele	39 (0.135)	44 (0.183)		1.433	0.896–2.293	0.1

OR Odds ratio, *CI* Confidence interval; * *P* value ≤ 0.05 significant; ** *P* value ≤ 0.01 highly significant. [a] *P* value is depending on the X^2 test, while [b] *P* value is depending on the logistic regression analysis

Table 3 Association of HCC characteristics with genotype distribution of the studied genes

		Total	PNPLA3 rs738409			*P* value	TM6SF2 rs58542926			*P* value
			CC	CG	GG		CC	CT	TT	
Age	<65	77 (64.2%)	31 (40.3%)	35 (45.5%)	11 (14.3%)	0.1	55 (71.4%)	17 (22.1%)	5 (6.5%)	0.9
	>65	43 (35.8%)	26 (60.5%)	13 (30.2%) [aa]	4 (9.3%) [aa, bb]		29 (67.4%)	11 (25.6%) [aa]	3 (7.0%) [aa, bb]	
Sex	Female	30 (25.0%)	13 (43.3%)	14 (46.7%)	3 (10.0%)	0.7	20 (66.7%)	8 (26.7%)	2 (6.7%)	0.9
	Male	90 (75.0%)	44 (48.9%)	34 (37.8%) [a]	12 (13.3%) [aa, bb]		64 (71.1%)	20 (22.2%) [aa]	6 (6.7%) [aa, bb]	
Diabetes mellitus (DM)		33 (27.5%)	14 (42.4%)	17 (51.5%)	2 (6.1%) [aa, bb]	0.2	25 (75.8%)	7 (21.2%) [aa]	1 (3.0%) [aa, bb]	0.6
F.H of HCC	No	117 (97.5%)	55 (47.0%)	48 (41.0%)	14 (12.0%)	0.3	81 (69.2%)	28 (23.9%)	8 (6.8%)	0.5
	Yes	3 (2.5%)	2 (66.7%)	0 (0.0%) [aa]	1 (33.3%) [aa, bb]		3 (100.0%)	0 (0.0%) [aa]	0 (0.0%) [aa]	
ECOG Performance status	0	75 (62.5%)	34 (45.3%)	34 (45.3%)	7 (9.3%) [aa, bb]	0.4	51 (68.0%)	17 (22.7%) [aa]	7 (9.3%) [aa, bb]	0.8
	1	40 (33.3%)	20 (50.0%)	12 (30.0%) [a]	8 (20.0%) [aa, b]		29 (72.5%)	10 (25.0%) [aa]	1 (2.5%) [aa, bb]	
	2	4 (3.3%)	2 (50.0%)	2 (50.0%)	0 (0.0%) [aa, bb]		3 (75.0%)	1 (25.0%) [aa]	0 (0.0%) [aa, bb]	
	3	1 (0.8%)	1 (100.0%)	0 (0.0%) [a]	0 (0.0%) [a]		1 (100.0%)	0 (0.0%) [a]	0 (0.0%) [a]	
Liver: size	Average	111 (92.5%)	52 (46.8%)	45 (40.5%)	14 (12.6%) [aa, bb]	0.7	77 (69.4%)	28 (25.2%) [aa]	6 (5.4%) [aa, bb]	0.2
	Enlarged	8 (6.7%)	5 (62.5%)	2 (25.0%) [aa]	1 (12.5%) [aa, b]		6 (75.0%)	0 (0.0%) [aa]	2 (25.0%) [aa, bb]	
	Shrunken	1 (0.8%)	0 (0.0%)	1 (100.0%) [a]	0 (0.0%) [b]		1 (100.0%)	0 (0.0%) [a]	0 (0.0%) [a]	
Spleen	Normal	46 (38.3%)	17 (37.0%)	23 (50.0%) [a]	6 (13.0%) [aa, bb]	0.3	36 (78.3%)	6 (13.0%) [aa]	4 (8.7%) [aa]	0.08
	Average	36 (30.0%)	19 (52.8%)	11 (30.6%) [aa]	6 (16.7%) [aa, bb]		21 (58.3%)	14 (38.9%) [aa]	1 (2.8%) [aa, bb]	
	Enlarged	38 (31.7%)	21 (55.3%)	14 (36.8%) [aa]	3 (7.9%) [aa, bb]		27 (71.1%)	8 (21.1%) [aa]	3 (7.9%) [aa, bb]	
Ascites	No	96 (80.0%)	47 (49.0%)	35 (36.5%)	14 (14.6%)	0.2	66 (68.8%)	24 (25.0%)	6 (6.3%)	0.7
	Yes	24 (20.0%)	10 (41.7%)	13 (54.2%) [a]	1 (4.2%) [aa, bb]		18 (75.0%)	4 (16.7%) [aa]	2 (8.3%) [aa, bb]	
CHILD Score	A	93 (77.5%)	45 (48.4%)	35 (37.6%) [a]	13 (14.0%) [aa, bb]	0.4	64 (68.8%)	22 (23.7%) [aa]	7 (7.5%) [aa, bb]	0.8
	B	25 (20.8%)	10 (40.0%)	13 (52.0%) [a]	2 (8.0%) [aa, bb]		18 (72.0%)	6 (24.0%) [aa]	1 (4.0%) [aa, bb]	
	C	2 (1.7%)	2 (100.0%)	0 (0.0%) [a]	0 (0.0%) [a]		2 (100.0%)	0 (0.0%) [a]	0 (0.0%) [a]	
CHILD grade		5.9±1.2	5.88±1.27	5.96±1.11	5.67±0.90	0.7	5.99±1.26	5.71±0.81	5.38±1.06	0.2
No. of F.L	No	2 (1.7%)	1 (50.0%)	1 (50.0%)	0 (0.0%) [aa, bb]	0.4	2 (100.0%)	0 (0.0%) [a]	0 (0.0%) [a]	0.9
	Single	77 (64.2%)	36 (46.8%)	28 (36.4%) [a]	13 (16.9%) [aa, bb]		53 (68.8%)	19 (24.7%) [aa]	5 (6.5%) [aa, bb]	
	Multiple	41 (34.2%)	20 (48.8%)	19 (46.3%)	2 (4.9%) [aa, bb]		29 (70.7%)	9 (22.0%) [aa]	3 (7.3%) [aa, bb]	
F.L site	Right lobe	97 (80.8%)	47 (48.5%)	37 (38.1%) [a]	13 (13.4%) [aa, bb]	0.9	67 (69.1%)	24 (24.7%) [aa]	6 (6.2%) [aa, bb]	0.3
	Left lobe	10 (8.3%)	4 (40.0%)	5 (50.0%) [a]	1 (10.0%) [aa, bb]		7 (70.0%)	1 (10.0%) [aa]	2 (20.0%) [aa, b]	
	Both lobes	13 (10.8%)	6 (46.2%)	6 (46.2%)	1 (7.7%) [aa, bb]		10 (76.9%)	3 (23.1%) [aa]	0 (0.0%) [aa, bb]	
F.L size		4.9±3.3	4.83±2.76	4.59±2.35	6.38±6.52	0.1	4.99±3.71	4.88±2.31	4.39±2.15	0.8
P.V	Patent	110 (91.7%)	53 (48.2%)	43 (39.1%)	14 (12.7%) [aa, bb]	0.8	76 (69.1%)	26 (23.6%) [aa]	8 (7.3%) [aa, bb]	0.6
	Thrombosed	10 (8.3%)	4 (40.0%)	5 (50.0%) [a]	1 (10.0%) [aa, bb]		8 (80.0%)	2 (20.0%) [aa]	0 (0.0%) [aa, b]	
Lymph nodes	No	114 (95.0%)	54 (47.4%)	46 (40.4%)	14 (12.3%)	0.9	81 (71.1%)	25 (21.9%)	8 (7.0%)	0.3
	Yes	6 (5.0%)	3 (50.0%)	2 (33.3%) [aa]	1 (16.7%) [aa, bb]		3 (50.0%)	3 (50.0%)	0 (0.0%) [aa, bb]	

Table 3 (continued)

		Total	PNPLA3 rs738409			*P* value	TM6SF2 rs58542926			*P* value
			CC	CG	GG		CC	CT	TT	
liver Stiffness		31.8±23.8	31.81±22.92	32.23±24.34	30.44±27.26	0.9	31.5±22.99	32.17±24.77	32.74±30.4	0.9
CAP		223.4±74.4	238.05±70.53	214.49±77.87	198.92±72.46	0.1	218.5±76.23	231.52±61.50	236.75±98.16	0.6
Response to treatment modified RECIST criteria	Complete	73 (60.8%)	32 (43.8%)	33 (45.2%)	8 (11.0%) [aa, bb]	0.04*	52 (71.2%)	15 (20.5%) [aa]	6 (8.2%) [aa, bb]	0.01*
	Partial	13 (10.8%)	8 (61.5%)	3 (23.1%) [aa]	2 (15.4%) [aa, b]		10 (76.9%)	2 (15.4%) [aa]	1 (7.7%) [aa, b]	
	Progressive	13 (10.8%)	6 (46.2%)	6 (46.2%)	1 (7.7%) [aa, bb]		4 (30.8%)	9 (69.2%) [aa]	0 (0.0%) [aa, bb]	
	Stationary	16 (13.3%)	9 (56.3%)	5 (31.3%) [aa]	2 (12.5%) [aa, bb]		14 (87.5%)	1 (6.3%) [aa]	1 (6.3%) [aa]	
Clinical decompensation	No	99 (82.5%)	47 (47.5%)	38 (38.4%)	14 (14.1%)	0.4	67 (67.7%)	24 (24.2%)	8 (8.1%)	0.3
	Yes	21 (17.5%)	10 (47.6%)	10 (47.6%)	1 (4.8%) [aa, bb]		17 (81.0%)	4 (19.0%) [aa]	0 (0.0%)	
Developed new lesions or not	No	113 (94.2%)	54 (47.8%)	44 (38.9%)	15 (13.3%)	0.5	80 (70.8%)	26 (23.0%)	7 (6.2%)	0.6
	Yes	7 (5.8%)	3 (42.9%)	4 (57.1%) [a]	0 (0.0%) [aa, bb]		4 (57.1%)	2 (28.6%) [aa]	1 (14.3%) [aa, b]	

intermediate stages (0.17%) patients (Table 3). The BCLC staging did not show a significant difference in genotype and allele distribution of PNPLA3 rs738409, although there was a significant higher frequency of the GG genotype and the G allele in the advanced stage compared to the CC genotype and the C allele, respectively (Table 4).

Furthermore, we observed the significant association between PNPLA3 rs738409 and TMS6F2 rs58542926 variant and the achievement of complete response according to the modified RECIST criteria.

Analysis of risk factors associated with HCC in the studied individuals

We assessed risk factors for HCC in the studied population, which showed that male gender, smoking, AFP, and diabetes were statistically significant risk factors in a univariate analysis. But only diabetes mellitus was the independent factor associated with the development of HCC by multivariate regression analysis (Table 5).

Correlation of the SNPs with HCC survival

We examined the impact of different factors including the studied polymorphisms on HCC survival in the studied patients (Table 6). Results showed that only diabetes, HCC stage, performance status, and response to mRECIST were significantly associated with patient's survival (Fig. 1), while polymorphism in PNPLA3 rs738409 and TM6SF2 rs58542926 does not have significant relation with survival, and similarly were age, gender, smoking, CHILD score, and liver stiffness (Fig. 1)

Discussion

To the best of our knowledge, so far this is the first study investigating the correlation between *PNPLA3* rs738409 and TM6SF2 rs58542926 polymorphism and HCC in Egyptian HCV-induced HCC patients.

In Egypt, HCC is the fourth most common cancer [29] and a mounting incidence of HCC has been recorded based on hospital studies [30–33], and it is considered

Table 4 Association of BCLC staging with genotype distribution of the studied genes

		BCLC stages				Total	*P* value
		Early *N*=56	Intermediate *N*=44	Late *N*=16	Advanced *N*=4		
PNPLA3	**CC**	26 (46.4%)	21 (47.7%)	8 (50.0%)	2 (50.0%)	57 (47.5%)	0.8
	CG	21 (37.5%)	19 (43.2%)	7 (43.8%)	1 (25.0%) [aa]	48 (40.0%)	
	GG	9 (16.1%) [aa, bb]	4 (9.1%) [aa, bb]	1 (6.3%) [aa, bb]	1 (25.0%) [aa]	15 (12.5%)	
	C Allele	73 (0.652)	61 (0.693)	23 (0.719)	5 (0.625)	162 (0.675)	0.7
	G Allele	39 (0.348) [**]	27 (0.307) [**]	9 (0.281) [**]	3 (0.375) [**]	78 (0.325)	
TM6SF2	**CC**	41 (73.2%)	32 (72.7%)	7 (43.8%)	4 (100.0%)	84 (70.0%)	0.2
	CT	11 (19.6%) [aa]	9 (20.5%) [aa]	8 (50.0%)	0 (0.0%) [aa]	28 (23.3%)	
	TT	4 (7.1%) [aa, bb]	3 (6.8%) [aa, bb]	1 (6.3%) [aa, bb]	0 (0.0%) [aa]	8 (6.7%)	
	C Allele	93 (0.830)	73 (0.830)	22 (0.688)	8 (1.000)	196 (0.817)	0.03*
	T Allele	19 (0.170) [**]	15 (0.170) [**]	10 (0.313) [**]	0 (0.000)	44 (0.183)	

Genotyping distributions are represented as frequency and percent; the data were analyzed by X^2 test. [a]*P* value is significantly different compared with wild type. [b]*P* value is significantly different compared with hetero type.[*]*P* value is significantly different compared with alleles

Table 5 Univariate and multivariate regression analysis of the risk factors of HCC

	[a]OR	95% C.I		*P* value	[b]OR	95% C.I		*P* value
		Lower bound	Upper bound			Lower bound	Upper bound	
Gender (male)	2.918	1.723	4.941	0.001**	0.525	0.218	1.266	0.1
Smoker	3.671	1.861	7.242	0.001**	0.363	0.112	1.179	0.09
BMI	0.959	0.909	1.011	0.1	0.710	0.253	1.996	0.5
DM	1.721	.960	3.086	0.05*	1.016	1.006	1.026	0.001**
AFP	1.021	1.013	1.029	0.001**	0.247	0.019	3.176	0.1

Body mass index (BMI), diabetes mellitus (DM), and alpha-fetoprotein (AFP). *OR* odds ratio, *C.I* confidence interval, *P* value was calculated depending on logistic regression analysis. **P* value <0.05 is significant, ***P* value <0.01 is highly significant. [a]OR for univariate analysis, [b]OR for multivariate analysis

Table 6 Factors associated with survival in HCC patients

	Dead *F*(%)	Median (95% C.I) of the estimate survival time	Log rank (Mantel-Cox)	*P* value
Gender				
Female	16 (21.6%)	262.0 (218.8–305.1)	1.183	0.3
Male	58 (78.4%)	335.0 (247.9–422.1)		
Age				
<65	55 (74.3%)	307.0 (221.9–392.1)	0.031	0.8
>65	19 (25.7%)	350.0 (202.1–497.9)		
Smoker				
No	53 (71.6%)	350.0 (276.6–423.4)	0.06	0.81
Yes	21 (28.4%)	306.0 (129.6–482.4)		
Diabetes				
No	56 (75.7%)	367.0 (279.0–455.0)	3.957	0.04*
Yes	18 (24.3%)	241.0 (95.5–386.5)		
BCLC stages				
Early	32 (43.2%)	398.0 (368.9–427.1)	16.803	0.001**
Intermediate	27 (36.5%)	271.0 (174.3–367.7)		
Advanced	3 (4.1%)	187.0 (210–379.0)		
Late	12 (16.2%)	226.0 (47.8–404.2)		
ECOG performance status				
0	44 (59.5%)	367.0 (283.6–450.4)	13.277	0.004**
1	26 (35.1%)	249.0 (141.6–356.4)		
2	3 (4.1%)	148.0 (136.8–159.2)		
3	1 (1.4%)	67.0 (67.0–67.0)		
Child-Pugh Score				
A	52 (70.3%)	369.0 (265.4–472.6)	0.25	0.9
B	20 (27.0%)	214.0 (146.1–281.9)		
C	2 (2.7%)	141.0 (120.0–141.0)		
Response to treatment-modified RECIST criteria				
Not treated	1 (1.4%)	57.0 (57.0–57.0)	22.281	0.001**
Stationary	14 (18.9%)	208.0 (182.3–233.7)		
Partial response	7 (9.5%)	306.0 (193.1–418.9)		
Complete response	41 (55.4%)	394.0 (371.0–417.0)		
Progressive disease	11 (14.9%)	226.0 (91.1–360.9)		
Liver stiffness cutoff = 9.5				
<9.5	5 (9.6%)	384.0 (25.4–742.6)	0.003	0.9
>9.5	47 (90.4%)	307.0 (221.0–393.0)		
Liver stiffness cutoff =12.5				
<12.5	9 (17.3%)	394.0 (364.8–423.2)	0.01	0.9
>12.5	43 (82.7%)	306.0 (241.8–370.2)		
PNPLA3				
CC	37 (50.0%)	306.0 (229.7–382.3)	1.963	0.4
CG	29 (39.2%)	307.0 (196.2–417.8)		
GG	8 (10.8%)	394.0 (345.5–442.5)		
TM6SF2				
CC	56 (75.7%)	312.0 (235.0–389.0)	2.104	0.3
CT	15 (20.3%)	248.0 (130.6–365.4)		
TT	3 (4.1%)	426.0 (378.0–474.0)		

C.I Confidence interval, the data were analyzed by Kaplan-Meier test. **P* value <0.05 is significant, and ***P* value <0.01 is highly significant

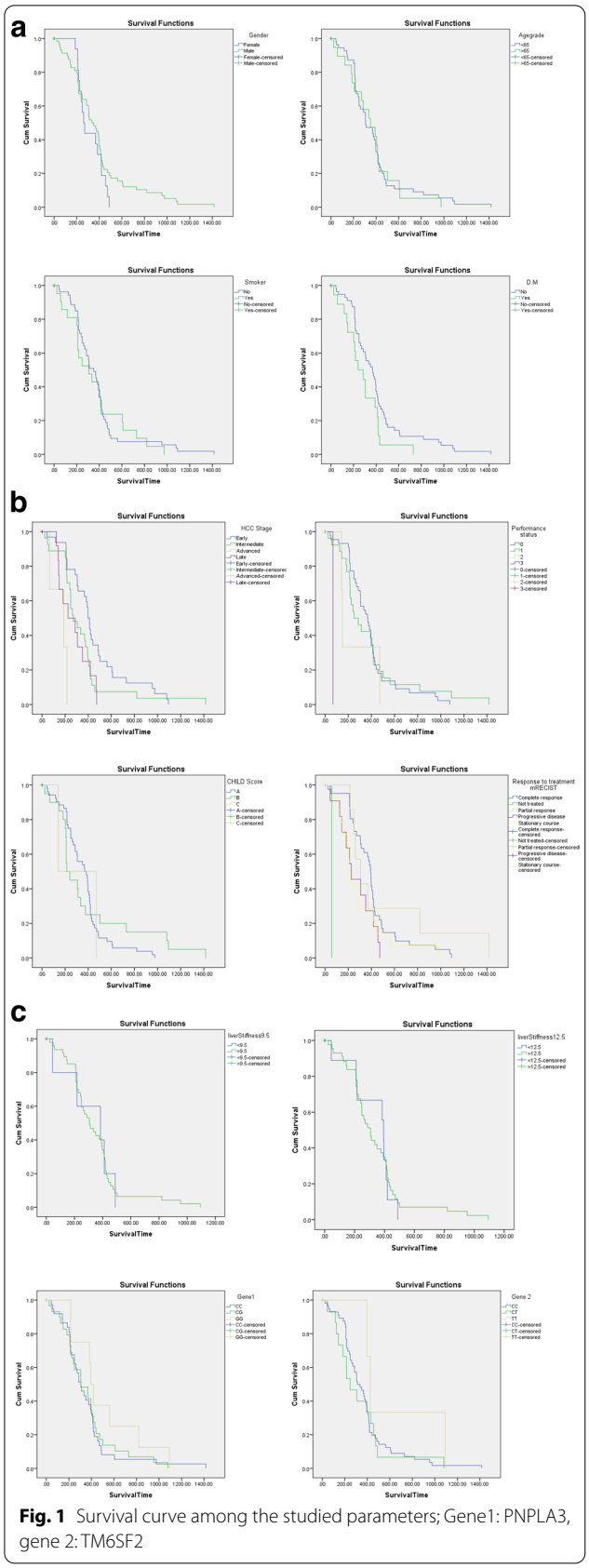

Fig. 1 Survival curve among the studied parameters; Gene1: PNPLA3, gene 2: TM6SF2

the most common cause of mortality and morbidity in Egypt [34].

It has been demonstrated that host genetic factors, such as single-nucleotide polymorphisms (SNPs), could affect individual susceptibility to HCC [35]. In this study, we explored the correlation between polymorphisms at PNPLA3 rs738409 and TM6SF2 rs58542926 and HCC in Egyptian patients who had HCV infection as the only etiological factor for HCC.

A single-nucleotide polymorphism rs738409 in *PNPLA3* is nowadays considered one of the genetic factors with an important impact on the progression of several liver diseases of different etiology [36]. In patients with ALD and NAFLD, the SNP was linked to an elevated risk of HCC in a meta-analysis of Western populations [36, 37]. This polymorphism has recently been associated with fibrosis advancement and liver carcinogenesis in patients with NAFLD in Asia [17, 38]. The PNPLA3 rs738409 polymorphism may be involved in hepatic steatosis and fibrosis in HCV patients, although its link to the development of HCC is less obvious, with mixed results [26, 36, 37, 39].

Our results showed that *PNPLA3* rs738409 polymorphism did not show an association with HCC development, this is controversial with Yang et al. [40] and Ezzikouri et al. [41] who showed an association between the PNPLA3 GG genotype and an increased risk of the HCC development and showed that patients with PNPLA3 GG genotype had a 3-fold increased risk when compared to PNPLA3 CC genotype in patients with mild chronic hepatitis C. This is a very interesting conflict as PNPLA3 rs738409 is known to exhibit ethnic diversity in its frequency [11, 42], so we expected similar results with Ezzikouri et al. (2014) study on Moroccan patients with similar Arabic ethnicity, but this was not the case and this may be attributed to the fact that Moroccan populations are mixed Berberic and Arabic ethnicity and that they reported a higher frequency (28%) of the risk GG genotype compared with only 12.5% in our Egyptian patients. Interestingly, on the contrary, our results agreed with results from Thai patients [34] who showed a PNPLA3 rs738409 GG frequency of 10.7% which is very similar to ours (12.5%) and showed this polymorphism is not linked to HCV-induced HCC. Our results are also in line with that of Ali et al. and Hai et al. [43, 44] representing mixed American plus European and Japanese races, respectively, who proved that PNPLA3 is not a significant risk factor for HCC among patients with HCV.

TM6SF2, mainly expressed in the liver, kidney, and gut tissue, is responsible for hepatic lipid metabolism by modulating triglyceride secretion and increased intracellular lipid droplet concentration [45]. Polymorphism in

TM6SF2 rs58542926 causes decreased protein expression, which is associated with higher intrahepatic triglyceride content and lower very low-density lipoprotein secretion [12]. The correlation between polymorphism in the TM6SF2 gene and the risk of liver cancer attracted many researchers' attention and results vary from one study to another.

TM6SF2 rs58542926, like PNPLA3 rs738409, contributes to the progression of liver disease in both NAFLD and ALD, ranging from steatosis to progressive fibrosis and cirrhosis [46]. In contrast, in a prior study from our team, we did not find a link between TM6SF2 rs58542926 polymorphism and the advancement of fibrosis in Egyptian HCV patients [47].

Recent reports from the European Caucasian populations demonstrated that the TM6SF2 T allele might be a potential genetic risk factor for developing HCC in patients with NAFLD and ALD [48]. Another study in Thai individuals showed that this variant was independently linked to non-hepatitis B non-hepatitis C (NBNC)-HCC, but not viral-induced HCC [34]. Results from a recent meta-analysis showed that the risk of liver cancer in the TT genotype group was significantly higher than that of the CC + CT genotype group [49].

It is uncertain whether the TM6SF2 rs58542926 allele increases the risk of HCC in HCV patients, and studies on the topic are scarce. Yang et al. confirmed for the first time in a prospective manner the link between TM6SF2 rs58542926 and HCC occurrence in a cohort of alcohol-related cirrhosis, but not associated with HCC development in HCV-related cirrhosis [40]. Our findings contradict those of Yang et al. since we found a link between the TT risk genotype and the development of HCC in Egyptian HCV-induced HCC patients. This disparity could be due to ethnic differences as well as HCV genotype differences. Indeed, more research on the link between TM6SF2 rs58542926 and HCV-induced HCC is urgently needed from people of various ethnicities and HCV genotypes in order to find out a scenario for this link. Consequently, genotyping of this polymorphism will allow more precise HCC risk stratification of patients with chronic liver diseases, and genotype-guided screening algorithms would optimize patient care [14].

Regarding the results of our study's correlation of these SNPs with clinical characteristics and prognostic significance, we discovered that PNPLA3 GG genotype patients had more advanced tumor stages than non-GG genotype patients, indicating that this polymorphism plays a prognostic role in the patients studied. This is in line with an Italian research that found patients with ALD- and NAFLD-related HCC who had the PNPLA3 GG genotype had more advanced tumor stages at presentation and worse survival than those who did not [50]. Similarly, we found a significant correlation between HCC stages and T allele of TM6SF2 rs58542926 pointing to the prognostic importance of this polymorphism also in HCV-induced HCC Egyptian patients, and this is a unique finding in our study and we did not reach mimicking finding in other studies. Unlike our results, Yang et al. [40] did not find any significant association between PNPLA3 rs738409, TM6SF2 rs58542926, and histological features of the tumor. Moreover, also Raksayot et al. [34] reported that the correlation of these SNPs with clinical characteristics and the prognostic significance was not observed in his cohort.

Limitations to our study include the lack of a healthy control group, and our aim mainly was to detect patients with the single etiology HCV-related HCC among the at-risk groups of chronic hepatitis C with cirrhosis. Another limitation to our study is the lack of matching in age and gender among cases of HCC and controls of chronic hepatitis C and cirrhosis. We included all patients who presented to our department (with chronic hepatitis C and cirrhosis and HCC) during the period of the study and follow-up, and then, we divided them into 2 groups with and without HCC as cases and controls, respectively. Patients with HCC are usually older and with male predominance and more deteriorated liver functions than patients with chronic hepatitis [2].

In addition, due to the success of the Egyptian campaign for HCV elimination in Egypt [51, 52], cases with chronic hepatitis C who did not receive DAAs became very small in number in the treating centers nowadays and patients who complete their treatment course and successfully eradicated the virus do not come for their follow-up, particularly old-aged patients.

In this study, we investigated also the correlation between PNPLA3 rs738409, TM6SF2 rs58542926 polymorphisms, and the survival of HCC patients studied, and we could not show a correlation between them. This is in line with previous studies [34], and a follow-up of patients with chronic hepatitis C even after complete viral clearance is highly recommended.

Conclusion

Our finding indicates that PNPLA3 and TM6SF2 variants might influence HCC development in this group of HCV-induced HCC Egyptian patients, suggesting differential mechanisms of liver carcinogenesis in relation to the underlying etiologies of liver disease and suggesting to consider the role of the hepatitis C genotype, in addition to emphasizing the importance of genotyping of these polymorphisms in HCV-risk patients for earlier detection and better management of HCC.

Abbreviations
PNPLA3: Patatin-like phospholipase domain-containing protein 3; TM6SF2: Transmembrane 6 superfamily member 2; HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus; SNPs: Single-nucleotide polymorphisms; NAFLD: Non-alcoholic fatty liver disease; MRI: Magnetic resonance imaging; RECIST: Response Evaluation Criteria in Solid Tumors.

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Authors' contributions
S.Y. did the study design. SY and E. A performed the experiment. S. Y and A. E wrote the first draft. S. S, M. N, H. S, and A. O did the clinical examination. The authors read and approved the final manuscript.

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Availability of data and materials
The authors confirm that the data supporting the findings of this study are available within the article.

Declarations

Ethics approval and consent to participate
This work was carried out following the Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments 1975 and its later amendments. The study protocol was approved by the ethical committee of the Faculty of Medicine, Cairo University, number (N-51-2018). All patients signed a written informed consent before inclusion in the study.

Consent for publication
The authors declare that they consent to the publication of this study.

Competing interests
The authors declare that they have no competing interests.

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