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 development of hepatocellular carcinoma (HCC). Aim: 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. Method: 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. Results: HCC Patients were significantly older with male predominance. A significant difference between TT genotype of TM6SF2 frequency was observed in HCC compared with HCV patients. Moreover, T allele of TM6SF2 distributions revealed significant contribution with different stages of HCC (p =0.03). Both PNPLA3 rs738409 and TM6SF2 rs58542926 variants showed 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. Diabetes, BCLC stage, performance status and response to treatment were significantly associated with patients' survival. Conclusion : TM6SF2 rs58542926 polymorphism not PNPLA3 rs738409 could be implicated in the development HCV induced HCC and its progression.

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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 development of hepatocellular carcinoma (HCC). **Aim:** 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. **Method:** 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. **Results:** HCC Patients were significantly older with male predominance. A significant difference between TT genotype of TM6SF2 frequency was observed in HCC compared with HCV patients. Moreover, T allele of TM6SF2 distributions revealed significant contribution with different stages of HCC (p=0.03). Both PNPLA3 rs738409 and TM6SF2 rs58542926 variants showed 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. Diabetes, BCLC stage, performance status and response to treatment were significantly associated with patients' survival. **Conclusion**: TM6SF2 rs58542926 polymorphism not PNPLA3 rs738409 could be implicated in the development 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 the 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 of liver cancer [5]. Genetic factors are related to the pathogenesis of liver cancer, and these 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 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 human. 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 level 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 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, Cipolla [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 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 264 subjects were recruited in this study including (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 from 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 was: (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, Alpha-fetoprotein (AFP) measurement 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 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 ^{0}C for genetic determinations.

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 analyse 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 were 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 2X TaqMan Universal MasterMix II, no UNG (Applied Biosystems, USA), 1.25 µl of Genotyping Assay 20X, 10.25 µl of Dnase free water (Promega, USA) and 1 µl of genomic DNA. The cycling was as follows: 95 ^{0}C for 10 min, followed by 40 cycles of 95 ^{0}C for 15 s and 60 ^{0}C for 1 min. The interpretation of genotypes for PNPLA3 rs738409 and TMS6F2 rs58542926 was given by (CC, CG, GG and CC, CT, TT respectively).

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%). Comparison between distributions of categorical variables was performed using Chisquare (X^2) test. In addition, variables were described as odds ratio (OR) with 95% confidence interval (95% CI) where appropriate. The data were considered significant t if the p value was < 0.05 and highly significant if $p < 0.01$. The associations between

the genes 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 in the difference in the patients' survival.

Results

Characteristics of the studied patients

A total of 264 subjects were analyzed in our study, including 120 patients with HCC, 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 HCC development in patients with HCV-related HCC (Table 2). Our data showed that the frequencies of PNPLA3 GG, CG, GG genotypes did not differ significantly between HCV and HCC patients. On the contrary, the frequencies of TM6SF2 TT genotype was 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 higher frequency of TM6SF2 T allele in late stage HCC patients (0.313%) compared to early and intermediate stages (0.17%) patients (Table 3). The BCLC staging did not show 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 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 age, male gender, smoking, AFP, and diabetes were statistically significant risk factor in univariate analysis. But only age and diabetes mellitus were the independent factors associated with the development of HCC by multivariate regression analysis.

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, 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 was age, gender, smoking, CHILD score and liver stiffness (Figure. 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 common cancer [26] and Mounting incidence of HCC have been recorded based on hospital studies [27], [28-30] and it is considered the most common cause for mortality and morbidity in Egypt [31].

It has been demonstrated that host genetic factors, such as single nucleotide polymorphisms (SNPs), could affect individual susceptibility to HCC[32]. 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.

Single nucleotide polymorphism rs738409 in *PNPLA3* is nowadays considered one of the genetic factors with an important impact on progression of several liver diseases of different etiology [33]. A met-analysis on Western populations showed that the SNP was associated with increased risk of HCC in patients with ALD and NAFLD [33, 34]. In Asia, it was recently reported also that this polymorphism was linked to fibrosis progression and liver carcinogenesis in patients with NAFLD [17, 35]. In patients with HCV infection, PNPLA3 rs738409 polymorphism might be involved in liver steatosis and fibrosis but its association with development of HCC was less clear with conflicting results [33, 34, 36, 37]

Our results showed that *PNPLA3* rs738409 polymorphism didn't show an association with HCC development, this is controversial with Yang, Trepo [38] and Ezzikouri, Alaoui [39] who showed an association between the PNPLA3 GG genotype and an increased risk of HCC development and showed that patients with PNPLA3 GG genotype had 3-fold increased risk when compared to PNPLA3 CC genotype in patients with mild chronic hepatitis C. This is very interesting conflict as PNPLA3 rs738409 is known to exhibit ethnic diversity in its frequency [11, 40], so we expected similar results with Ezzikouri and colleagues, 2014 study on Moroccan patients with similar Arabic ethnicity but this wasn't 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 [31] 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 [41, 42] 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 [43]. 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 TM6SF2 gene and the risk of liver cancer attracted many researcher's attention and results vary from study to the other.

Similar to PNPLA3 rs738409, TM6SF2 rs58542926 is contributable to the progressive liver disease not only NAFLD but also ALD, extending from steatosis to progressive fibrosis and cirrhosis [44]. On the contrary, our previous study showed that there wasn't a correlation between TM6SF2 rs58542926 polymorphism and fibrosis progression in Egyptian HCV patients [45]. Recent reports from European Caucasian populations demonstrated that TM6SF2 T allele might be a potential genetic risk factor for developing HCC in patients with NAFLD and ALD [46]. 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 [31] . Results from a recent met-analysis showed that the risk of liver cancer in the TT genotype group was significantly higher than that of the CC + CT genotype group [47].

It is unclear whether TM6SF2 rs58542926 increases the risk of HCC in HCV patients, and studies exploring this correlation are extremely few. Yang et al., Confirmed for the first time in a prospective manner the link between TM6SF2 rs58542926 and HCC occurrence in cohort of alcohol related cirrhosis, but not associated with HCC development in HCV-related cirrhosis [38]. Our data are contradictory with Yang et al; as our results showed a correlation between carrying the TT risk genotype and development of HCC in Egyptian HCV induced HCC patients. This difference may be attributed to different ethnicity in addition to different HCV genotype. Indeed, further studies dissecting this correlation between TM6SF2 rs58542926 and HCV induced HCC are urgently needed from different ethnicities and HCV genotypes in order to be able to figure a scenario for this correlation. 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 results of the correlation of these SNPs with clinical characteristics and the prognostic significance in our study, we found that PNPLA3 GG genotype had more advanced tumor stages than non GG genotype patients indicating a prognostic role of this polymorphism in the studied patients, this is concordant with an Italian study which reported that patients with ALD- and NAFLD-related HCC harboring PNPLA3 GG genotype had more advanced tumor stages at presentation and worse survival compared with individuals with non-GG genotype [48]. Similarly, we found a significant correlation between HCC stages and T allele of TM6SF2 rs58542926 pointing the prognostic importance of this polymorphism also in HCV induced HCC Egyptian patients and this is a unique finding to our study we did not reach mimicking finding in other studies. In contrast to our results, [38] did not identify any significant association between PNPLA3 rs738409, TM6SF2 rs58542926 and histological features of the tumor. Moreover, also [31] reported that the correlation of these SNPs with clinical characteristics and the prognostic significance was not observed in his cohort.

In this study we investigated also the correlation between PNPLA3 rs738409, TM6SF2 rs58542926 polymorphisms and survival of HCC patients studied and we couldn't show a correlation between them. This is in line with the study of [31]

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.

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Disclosure statement

Authors declare that they have no conflict of interest regarding the publication of this paper.

Ethical approval

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).

Consent to participate

All patients signed a written informed consent before inclusion in the study.

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Availability of data

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

Author contributions

All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission

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Tables

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), Creatinine (Crea) are represented as Mean ± SD; the data were analyzed by student t test. While ALT, AST, Total bilirubin (Bil.T) and AFP are represented as 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 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]	
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]	

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 comparing with Wild type. [b] p value is significantly different comparing with Hetero type.[*] p value is significantly different comparing between Alleles .

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

	[1]OR	95% C.I		P. value	[2]OR	95% C.I		P. value
		Lower Bound	Upper Bound			Lower Bound	Upper Bound	
Age	1.277	1.202	1.357	0.001**	1.251	1.170	1.338	0.001**
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; Odd Ratio, C.I; Confidence Interval, p-value calculated depend on logistic regression analysis. * p. value <0.05 is significant, ** p. value <0.01 is highly significant. [1]OR for univariate analysis, [2]OR for multivariate analysis.

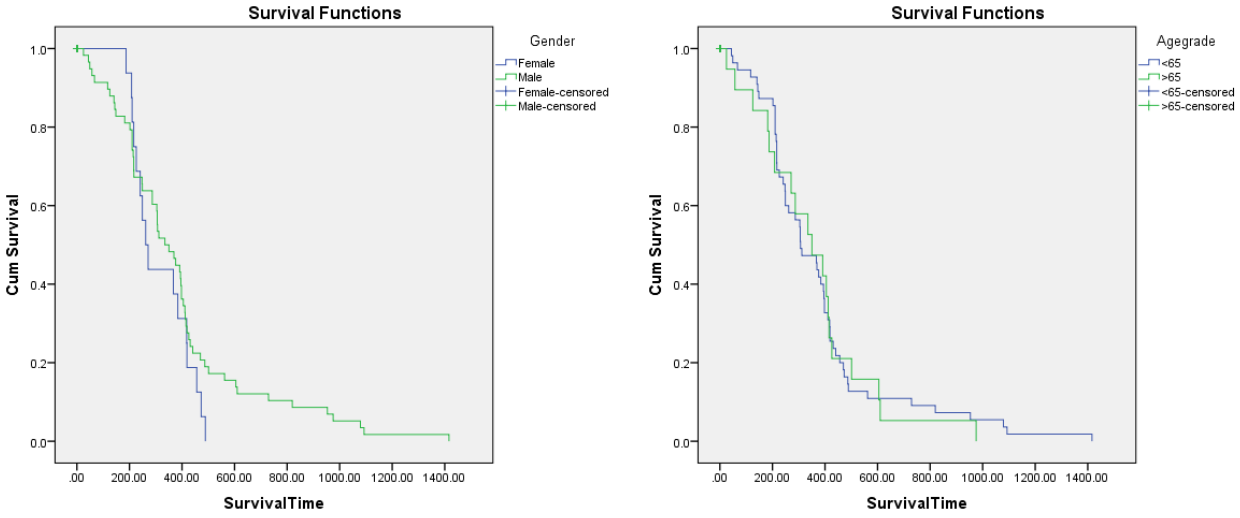
Table 6: Factors associated with survival in HCC patients

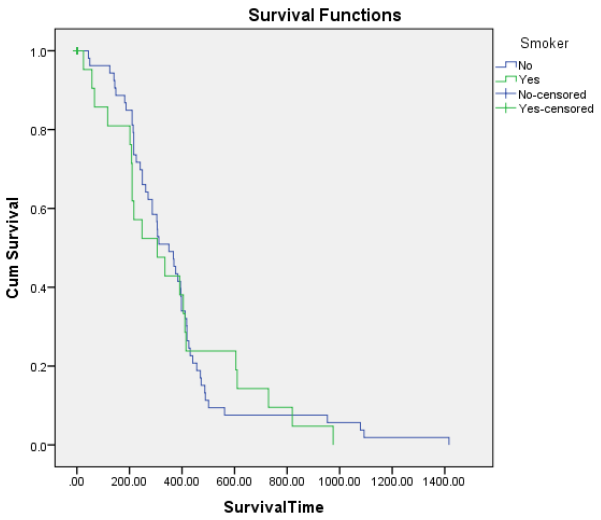
		Dead F(%)	Median (95% C.I) of the Estimate Survival Time	Log Rank (Mantel-Cox)	P. value
Gender	Female	16(21.6%)	262.0(218.8 - 305.1)	1.183	0.3
	Male	58(78.4%)	335.0(247.9 - 422.1)		
Age	<65	55(74.3%)	307.0(221.9- 392.1)	0.031	0.8
	>65	19(25.7%)	350.0(202.1- 497.9)		
Smoker	No	53(71.6%)	350.0(276.6- 423.4)	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 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, ** p. value <0.01 is highly significant

Figures




Survival Functions
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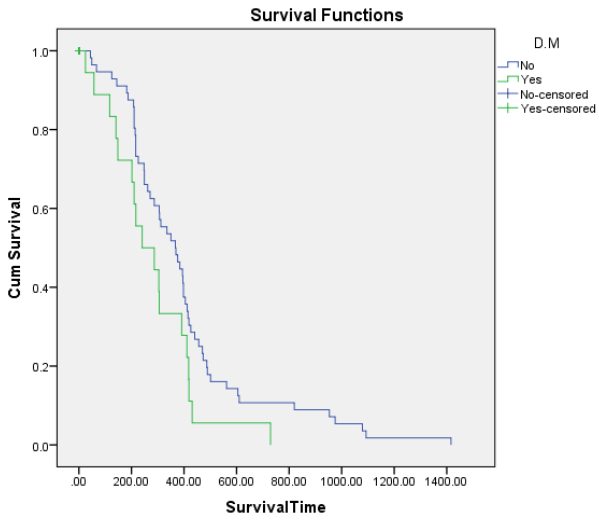
Yes
No-censored
Yes-censored
Cum Survival
SurvivalTime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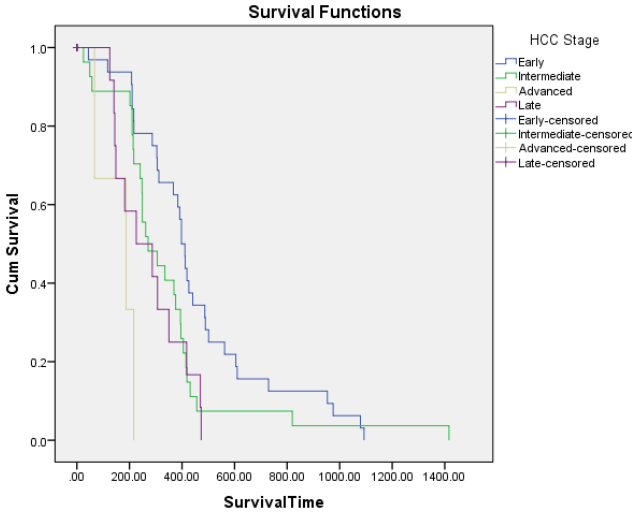
SurvivalTime


Survival Functions
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Intermediate
Advanced
Late
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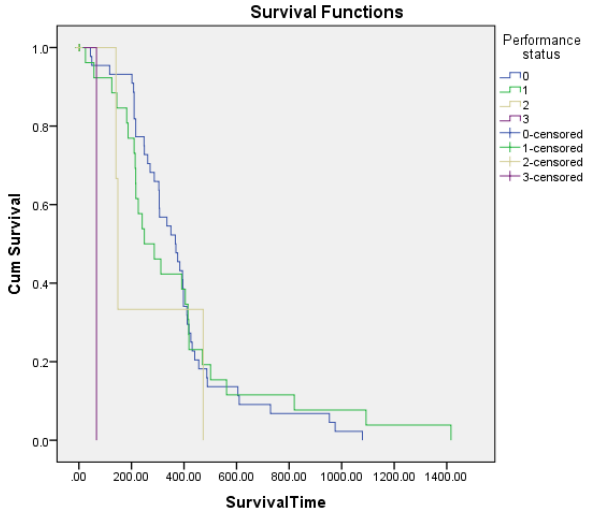
Advanced-censored
Late-censored
Cum Survival
SurvivalTime


Survival Functions
Performance status
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2
3
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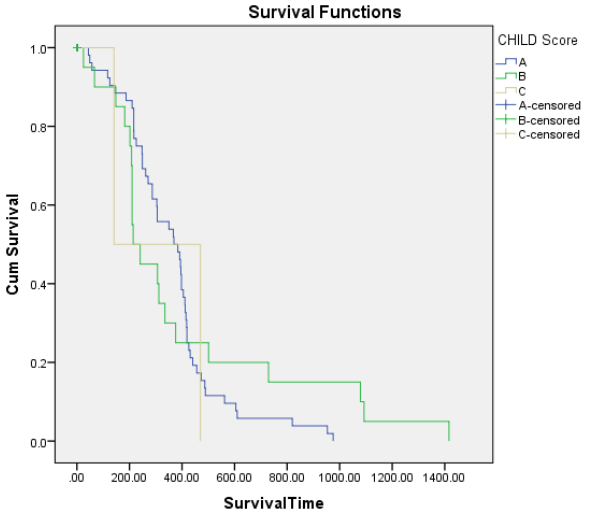
1-censored
2-censored
3-censored
Cum Survival
SurvivalTime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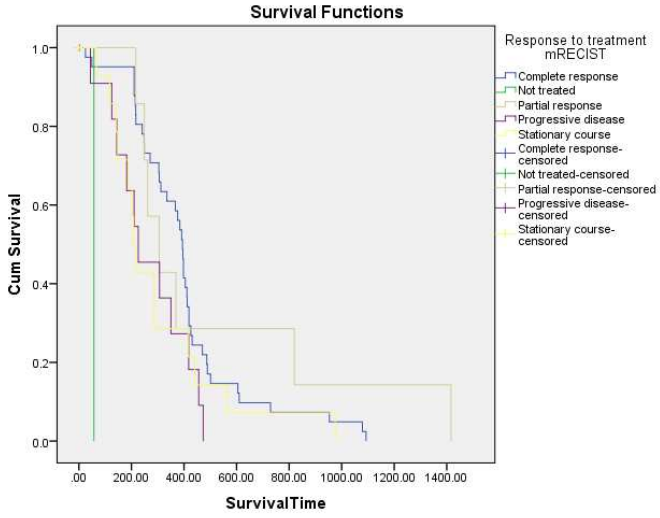
A
B
C
A-censored
B-censored
C-censored
Cum Survival
SurvivalTime


Survival Functions
Response to treatment mRECIST
Complete response
Not treated
Partial response
Progressive disease
Stationary course
Complete response-censored
Not treated-censored
Partial response-censored
Progressive disease-censored
Stationary course-censored
Cum Survival
SurvivalTime

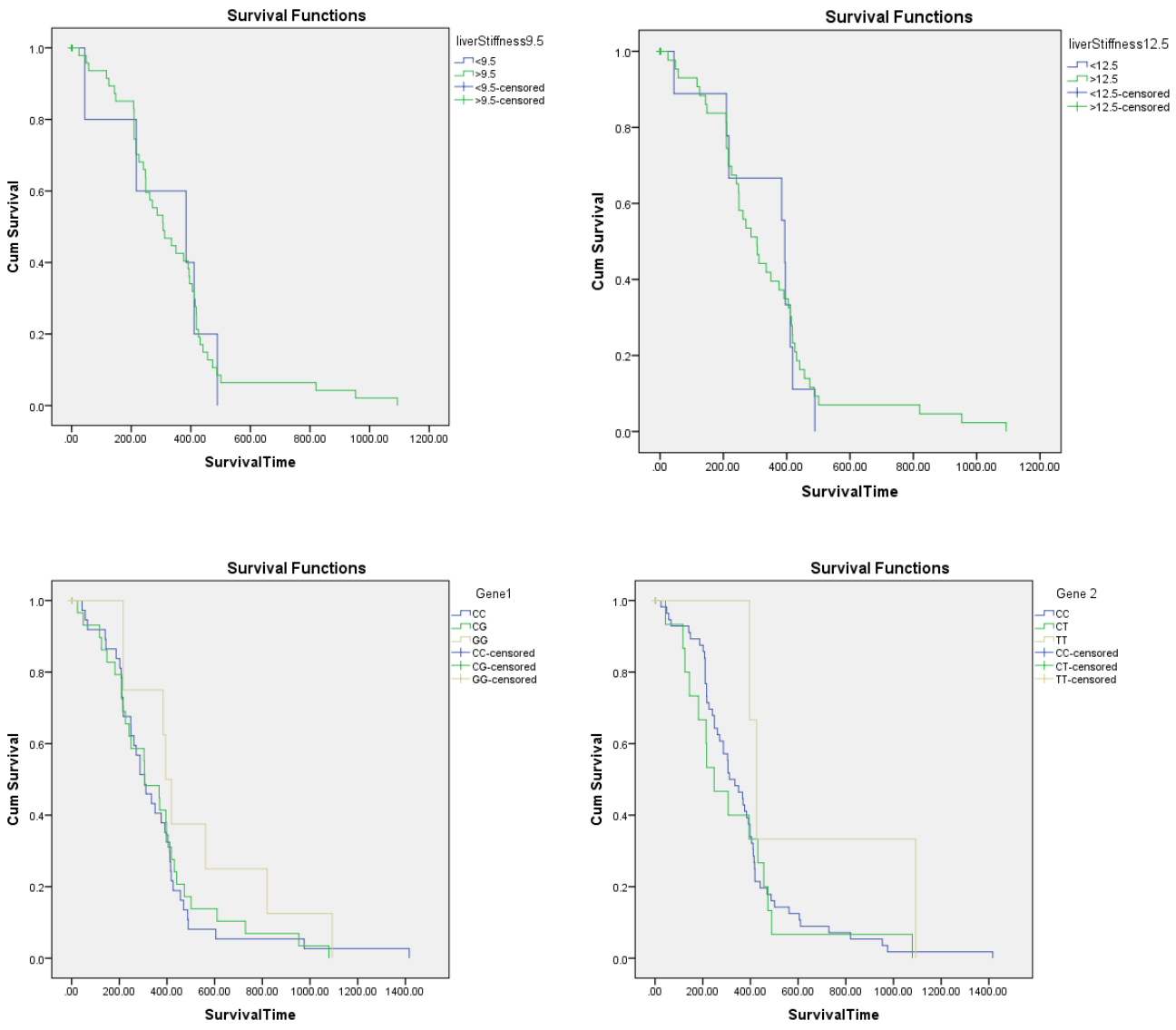


Fig. 1: Survival curve among the studied parameters; Gene1: PNPLA3, gene 2: TM6SF2

Figures

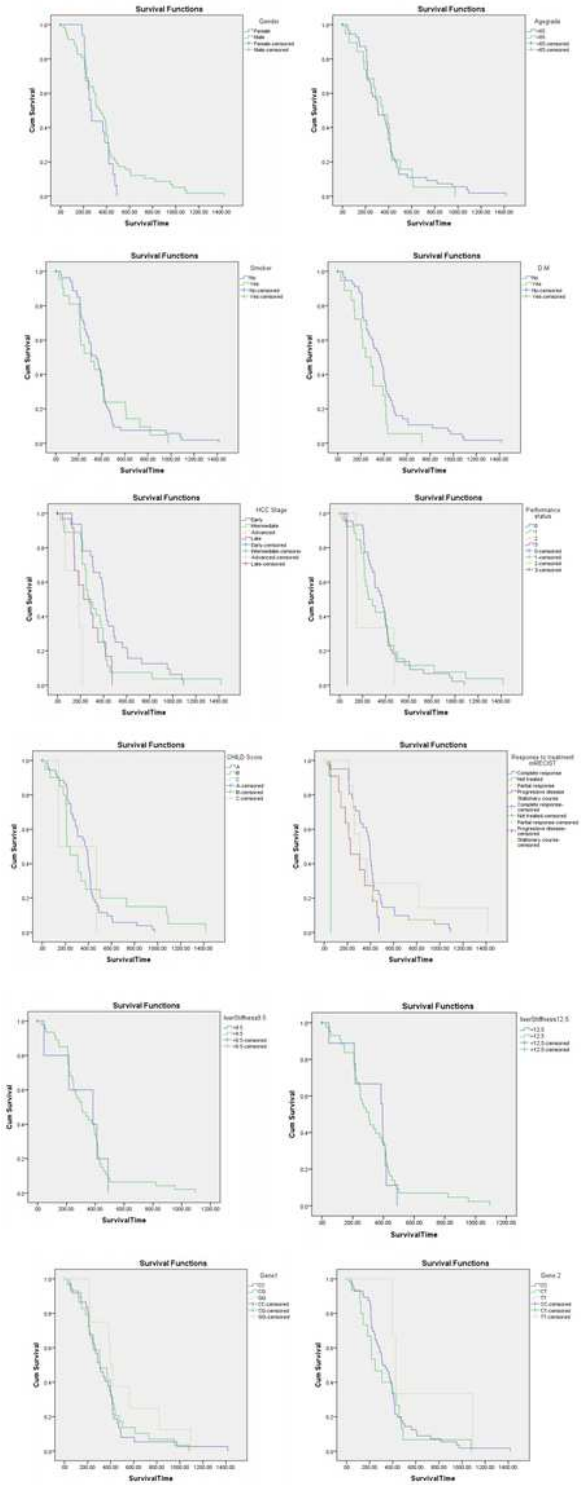


Figure 1

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