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HCV genotype 3 is associated with a higher hepatocellular carcinoma incidence in patients with ongoing viral C cirrhosis

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SUMMARY. Liver steatosis is a main histopathological feature of Hepatitis C (HCV) infection because of genotype 3. Steatosis and/or mechanisms underlying steatogenesis can contribute to hepatocarcinogenesis. The aim of this retrospective study was to assess the impact of infection with HCV genotype 3 on hepatocellular carcinoma (HCC) occurrence in patients with ongoing HCV cirrhosis. Three hundred and fifty-three consecutive patients (193 men, mean age 58 ± 13 years), with histologically proven HCV cirrhosis and persistent viral replication prospectively followed and screened for HCC between 1994 and 2007. Log-rank test and Cox model were used to compare the actuarial incidence of HCC between genotype subgroups. The patients infected with a genotype 3 (n = 25) as compared with those infected with other genotypes (n = 328) had a lower prothrombin activity [78 (interquartile range 60–85) *vs* 84 (71–195) %, P = 0.03] and higher rate of alcohol abuse (48% *vs* 29%, P = 0.046). During a median follow-up of 5.54 years [2.9–8.6], 11/25 patients (44%) and 87/328 patients (26%) with a genotype 3 and non-3 genotype, respectively, develop a HCC. HCC incidences were significantly different among the genotype subgroups (P = 0.001). The 5-year occurrence rate of HCC was 34% (95% CI, 1.3–6.3) and 17% (95% CI, 5.7–9.2) in genotype 3 and non-3 genotype groups, respectively (P = 0.002). In multivariate analysis, infection with a genotype 3 was independently associated with an increased risk of HCC occurrence [hazard ratio 3.54 (95% CI, 1.84–6.81), P = 0.0002], even after adjustment for prothrombin activity and alcohol abuse [3.58 (1.80–7.13); P = 0.003]. For patients with HCV cirrhosis and ongoing infection, infection with genotype 3 is independently associated with an increased risk of HCC development.

Keywords: HCV genotype 3, hepatocellular carcinoma, steatosis.

INTRODUCTION

Infection with HCV genotype 3 is characterized by a high rate of viral eradication after antiviral treatment and by liver steatosis called viral steatosis. In this condition, steatosis can occur in the absence of predisposing conditions such as overweight, diabetes mellitus or alcohol abuse, is correlated with the serum viral load, decreases after viral eradication and reappears after relapse [1–4]. HCV genotype 3 induces increased triglyceride accumulation in the hepatocytes by two potential mechanisms: impaired excretion of lipids from the infected hepatocytes resulting from a decreased intrahepatic activity of microsomal triglyceride transfer protein [5] and by increased lipids biosynthesis by activation of fatty acids synthase (FAS) promoter, sterol regulatory element-binding proteins (SREBP) by genotype 3 core protein [6,7]. Amount of experimental data support a link between hepatic steatosis and hepatocellular carcinoma (HCC) development. Increased species reactive oxygen production, lipid peroxidation and accelerated hepatocyte proliferation were observed in steatotic livers [8–10]. Furthermore, pathways involved in steatogenesis such as SREBP or FAS activation may also participate in liver carcinogenesis [11]. In models of transgenic mice expressing the core protein, the structural and nonstructural proteins of HCV, or in genetically obese leptin-deficient ob/ob mice, HCC is preceded by steatosis and occurs in the absence of inflammation and fibrosis [12–14]. In human, steatosis is frequently observed in the nontumorous liver of noncirrhotic patients who develop HCC [15,16]. In some studies, infection with HCV genotype 3 was

Abbreviations: CHC, chronic hepatitis C; HCC, hepatocellular carcinoma; HCV, hepatitis C virus.

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associated with faster fibrosis progression and higher degree of portal hypertension [17,18]. But up to now, an increased incidence of HCC in cirrhotic patients infected with genotype 3 has not been documented. This study was designed to evaluate the impact of infection with a genotype 3 on HCC occurrence in a large cohort of patients with HCV cirrhosis and persistent viral replication prospectively followed and screened for HCC.

PATIENTS

Inclusion criteria

We retrospectively analysed the prospectively collected data of a cohort of patients included in a screening programme for HCC detection between 1994 and Jan 2007 satisfying the following criteria: (i) a compensated (Child A5 or 6), histologically proven cirrhosis (METAVIR F4) and absence of detectable or suspected HCC, (ii) presence of anti HCV antibodies and detectable HCV RNA by reverse transcription polymerase chain reaction serum in the serum as well as a genotyping of the virus, (iii) absence of HBV or HIV infections, hemochromatosis, biliary cirrhosis, Wilson's disease and alpha 1 antitrypsin deficiency and (iv) no severe life-threatening disease. Patients who achieved a sustained virological response after antiviral treatment during the follow-up period were excluded.

Patients

All patients had histologically proven cirrhosis with antibodies against HCV (Monolisa anti HCV; Sanofi Diagnostics Pasteur, Marnes la coquette, France) and detectable HCV RNA by PCR (Amplicor HCV; Roche Diagnostics, Branchburg, NJ, USA). HCV genotyping was performed before any antiviral treatment, using a second-generation reverse hybridization line probe assay (Inno-Lipa HCV II; Innogenetics, Zwijndrecht, Belgium).

Methods

Baseline clinical and biological parameters were recorded at the date of inclusion in the screening programme for HCC detection.

Diabetes status was collected as a binary parameter (yes/no) and was defined by fasting serum glucose level >126 mg/dL or by a previous anti diabetic treatment. BMI was calculated as weight (kg) divided by height (m) squared (kg/m^2).

Liver steatosis

Macrovacuolar steatosis was evaluated on liver specimens obtained for diagnosis of cirrhosis and fixed in formalin, embedded in paraffin and stained with haematoxylin–eosin and Masson's trichrome. All liver biopsy specimens were reviewed by an experienced hepatopathologist (MZ) unaware of clinical and biological data. Macrovacuolar steatosis was graded as the percentage of hepatocytes containing macrovacuolar fat droplets in three classes: <10%, 10–30% and ≥30% of hepatocytes affected. Only biopsies more than 10 mm in length were scored.

Follow-up

After inclusion, patients were followed up at least every 6 months and were screened for HCCs by abdominal ultrasonography and serum alphafoetoprotein (AFP) levels every 6 months. Diagnosis of HCC was based on histology or on noninvasive criteria according to EASL recommendations [19].

Statistical analysis

Clinical data of all patients were prospectively collected in a computerized database. Baseline continuous variables were expressed as means ± SD, medians and interquartile ranges or percentages as appropriate. Comparison between groups used Mann–Whitney test for quantitative data and X test or Fisher exact test for qualitative data. Survival curves were calculated using the Kaplan–Meier method and compared using the log-rank test. Cox regression models were used to compare the actuarial incidence of HCC between genotype subgroups. All variables found to be significant ($P < 0.10$) at the univariate analysis were entered into a multivariate stepwise model. Data were censored when a patient died, was transplanted without previous diagnosis of HCC, was lost of follow-up, or was at the last visit until December 2007. The hazard rates were reported with 95% CIs. All analyses were two-sided, and P values <0.05 were considered statistically significant. Analyses were conducted using SAS (version 9.2.; SAS Institute, Cary, NC, USA).

RESULTS

Baseline characteristics

Among 425 patients with HCV cirrhosis and no HCC prospectively screened, we excluded 15 patients without available genotype, five who had a genotype 5 ($n = 3$) or 6 ($n = 2$). Two hundred and two patients received more than 6 months of antiviral treatment. Among them, 52 patients [infected with genotype 1 ($n = 25$), genotype 2 ($n = 9$), genotype 3 ($n = 10$) and genotype 4 ($n = 8$)] achieved a sustained virological response after one or more treatment courses and were excluded. Therefore, 353 patients were included in this study (Fig. 1). Table 1 summarizes the main baseline characteristics of the overall population. Two hundred and fifty-one (71%) had genotype 1, 33 (10%) had genotype 2, 25 (7%) had genotype 3 and 44 (12%) had genotype 4. There was a significant difference regarding baseline prothrombin activity

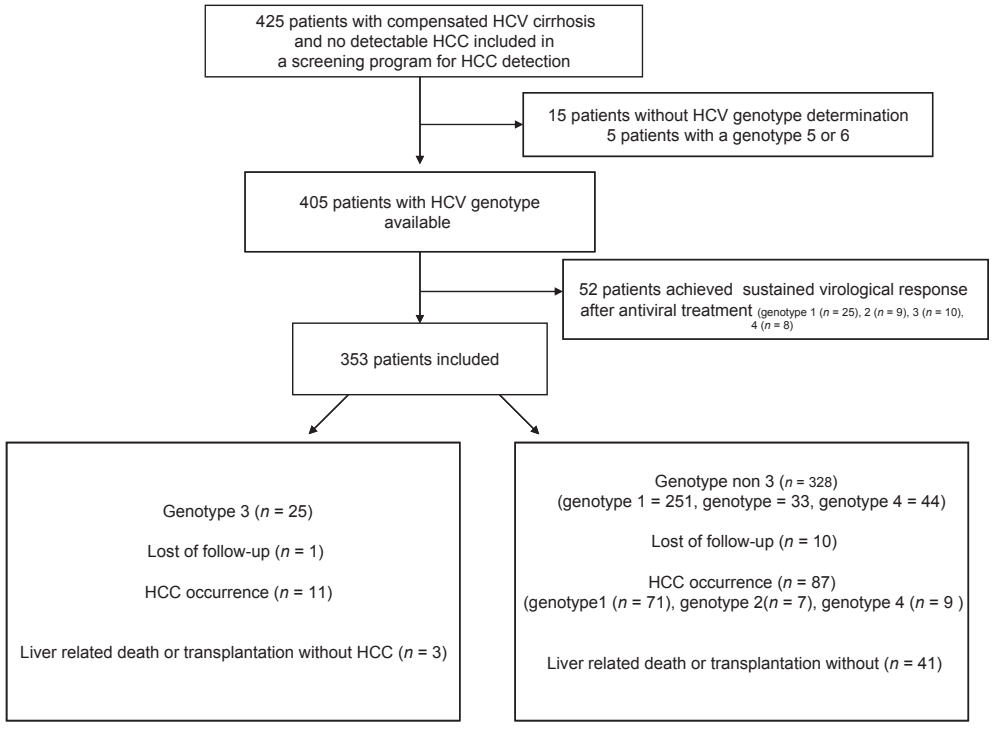

Fig. 1 Patient's enrolment and outcomes.

Table 1 Baseline patient's characteristics according to HCV genotype 3 or non-3 genotype

Variables	Patients infected with HCV genotype 3 (n = 25)	Patients infected with HCV non-3 genotype (n = 328)	P value
Age mean (year) (SD)	53.82 (41.85; 67.71)	58. 21 (47.75; 68.43)	0.14
Male gender (n, %)	15 (60)	178 (54.3)	0.58
Past and or daily ethanol intake >30 g/day (%)	12 (48)	95 (29)	0.045
Diabetes (n, %)	7 (28)	106 (33)	0.61
BMI (kg/m^2) ± SD	24.89 (23.51; 27.5)	24.98 (22.91; 28.41)	0.90
ALT (XN)	2 (2; 3)	2.5 (1.5; 3.7)	0.84
Platelet count (×10^9/L) (SD)	108 (79; 149.5)	127.5 (90; 171.5)	0.22
Prothrombin activity (%) (SD)	78 (60; 85)	84 (71; 95)	0.035
Serum albumin (g/L)	44.5 (38; 47)	41 (38; 45)	0.19
Serum bilirubin (μM/L) ± SD	13.5 (8; 21)	13 (10; 20)	0.88
Serum AFP (ng/mL) (SD)	8 (5; 14)	8 (5; 14)	0.99
HCV viral load (×10^6 UI/mL	1.0 (0.4; 2.7)	0.8 (0.5; 2.9)	0.7
Grade of steatosis (% of hepatocytes)†			0.06
<10	10 (40)	199 (63)	0.026
10–30	8 (32)	66 (21)	
≥30	7 (28)	50 (16)	
Steatosis >10	15 (60)	118 (37)	

Median (q1–q3) for continuous variables, ALT, alanine transferase; ULN (upper limit range, Non-3 genotype included genotype 1 = 251, genotype 2 = 33, genotype 4 = 44), †assessed in 340 patients (25 with genotype 3 and 315 with non-3 genotype); available in 350 patients (22 with genotype 3 and 278 with non-3 genotype).

(P = 0.035) and past or ongoing alcohol intake (P = 0.046), but not for age, sex, BMI and platelet count between patients infected or not with a genotype 3.

Liver biopsies specimen allowing a reliable grading of macrovacuolar steatosis was available for 340 patients. Steatosis was graded as follow: <10% (grade 0) in 209 cases

Table 2 Comparison of patients characteristics between patients with liver steatosis <10% and ≥30%

Variables	Patients in with steatosis ≥30% (n = 57)	Patients in with steatosis <10–30% (n = 74)	Patients in with steatosis <10% (n = 209)	P value
Male gender (n, %)	25 (45.1)	37 (51.4)	117 (56.7)	0.309
Genotype 3 (%)	7 (4.78)	8 (10.8)	7 '13.7)	0.058
Past and/or daily ethanol intake >30 g/day (%)	17 (31.3)	20 (27.9)	63 (29.7)	0.91
Diabetes (%)	17 (31.3)	27 (27.9)	60 (29.7)	0.43
Age mean (year) (SD)	60.49 (47.69; 68.77)	58.26 (46.38; 67.5)	58.09 (47.71; 66.96)	0.79
BMI (kg/m^2) ± SD	25.74 (24.03; 29.32)	25.4 (23.29; 29.1)	24.61 (22.2; 27.4)	0.06
ALT (XN)	3 (1.65; 4.5)	2.4 (1.8; 3.65)	2.2 (1.5; 3.5)	0.52
Platelet count ×10^9/L)	140 (97; 182)	122 (94; 171)	120 (86; 169)	0.24
Prothrombin activity (%) (SD)	86 (78; 99)	81 (70; 91)	82.5 (68; 91)	0.10
Albumin (g/L)	44 (38; 47)	42 (38; 46)	40 (37; 44)	0.056
Total bilirubin (μM/L) ± SD	11 (10; 18)	13 (10; 17)	14 (9; 22)	0.48
Hepatocellular carcinoma (%)	14 (25)	24 (33)	58 (28)	0.56
AFP (ng/mL) (SD)	10 (6; 15)	9.5 (6; 17)	7 (4; 12)	0.0028

For qualitative variables: n (percentage), median (q1–q3) for continuous variables.

(61%); 10–30% (grade 1) in 73 cases (22%); and ≥30% (grade 2) in 57 cases (17%).

The proportion of patients with steatosis >10% was significantly higher in patients infected with a genotype 3 compared with patients infected with a nongenotype 3; 61% *vs* 37% (P = 0.035). In comparison with patients with steatosis <10%, patients with steatosis ≥30% had a lower rate of thrombopenia (platelet count ≤150 000/mm^3) (P = 0.032), and higher prothrombin activity (P = 0.039), serum albumin (P = 0.036), BMI (P = 0.035) and baseline serum AFP level (P = 0.016). Patients' characteristics according to the grade of steatosis are reported in Table 2.

Development of hepatocellular carcinoma

Eleven patients (one in the genotype 3 and 10 in the nongenotype 3 group) were lost of follow-up and censored at the date of the last visit. During a median follow-up of 5.54 years (2.9–8.6), 96 patients develop a HCC: 11/25 patients (44%), 71/251 patients (28%), 7/33 patients (21%) and 9/44 patients (20%) with a genotypes 3, 1, 2 and 4, respectively. The diagnosis of HCC was based on histology (n = 39) or on noninvasive criteria (n = 57).

Hepatocellular carcinoma-free survival according to HCV genotypes subgroup and between patients with genotype 3 or non-3 genotype is reported in Fig. 2. The 5-year occurrence rate of HCC was 34% (95% CI, 1.3–6.3) and 17% (95% CI, 5.7–9.22) for patients infected with genotype 3 and non-3 genotype, respectively (P = 0.013) (Fig. 3). Univariate Cox regression analysis showed that there was no significant association steatosis grade 2, grade of steatosis in two classes (< or >10%),or the different classes of

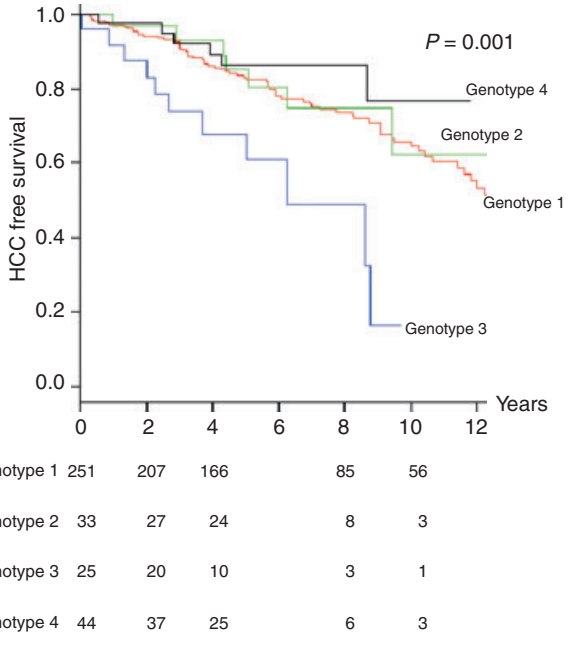

Fig. 2 Probabilities of HCC free survival according to HCV genotype (Kaplan Meier) P = 0.001.

steatosis (HR, 0.9 CI, 0.68–1.19) (P = 0.47) and HCC development.

Multivariate Cox regression analysis showed that infection with a genotype 3 along with male gender, older age, high BMI and low platelet count were significantly associated with HCC development (Table 3). Infection with genotype 3 was still associated with HCC occurrence after adjusting with prothrombin activity and alcohol abuse (HR, 3.58 CI, 1.80–7.13, P = 0.003).

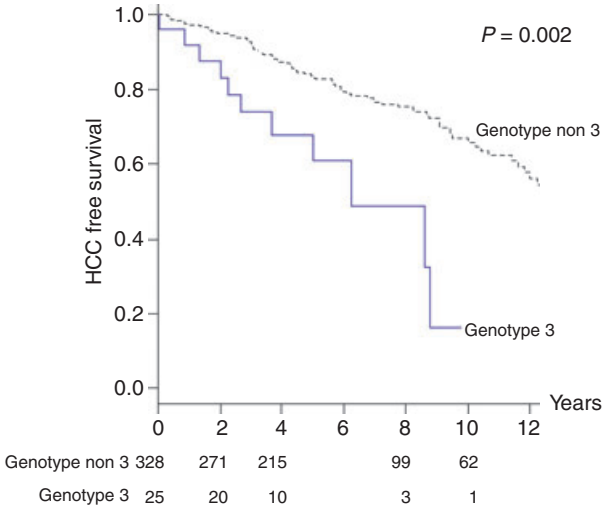

Fig. 3 Probabilities of HCC free survival according to HCV genotype 3 and non 3 (Kaplan Meier) $P = 0.002$.

DISCUSSION

In this retrospective study of a large cohort of patients with HCV cirrhosis and persistent viral replication prospectively screened for HCC, we observed that infection with a genotype 3 was associated with an increased risk of HCC occurrence either in univariate or in multivariate analysis taking in account the most commonly recognized risk factors. Conversely, no statistically significant difference in HCC incidence was observed between patients infected with genotype 1, 2 or 4. Our results confirmed the influence of known risk factors of HCC development, including male sex, older age, higher BMI and low platelet [20]. As HCV genotype 3 is considered as fairly responsive to antiviral therapy, our finding reinforces the need to achieve a sustained viral response (SVR) even using prolonged or repeated courses of treatment. Patients with cirrhosis are generally considered as difficult to treat and more prone to treatment complications. But in patients with genotype 3, the very high incidence of HCC must clearly counterbalance the fear of secondary effects.

The prevalence of genotype 3 infection in this study is lower than the main prevalence observed in the French population infected with HCV virus (around 20%) [21]. This bias could be explained by the higher response rate of this genotype to antiviral treatments that precludes the progression of liver disease to cirrhosis and by the fact we excluded patients who achieved a SVR during the follow-up, because viral clearance markedly reduces the risk of HCC development [22] and could therefore be considered as a bias. The overall low sustained responder's rate observed in our study was in part related to the fact that in the early period of the study, patients did not received pegylated interferon or bitherapy, and that cirrhosis present in all our patients is predictive of poor response. Conversely, the fact that nonresponders infected with a genotype 2 had a significantly lower risk of HCC in comparison with those with genotype 3 suggests that our results could not be explained only by the selection of patients bearing host factors of poor virological response. Furthermore, genotype 3 infection was still strongly associated with HCC occurrence when adjusted

Table 3 Univariate and multivariate analysis of Predictors for hepatocellular carcinoma development in 353 patients with compensated HCV cirrhosis

	Univariate analysis			Multivariate analysis		
Variables	HR	95% CI	*P* value	HR	95% CI	*P* value
Age mean (year) (SD)	1.03	1.01–1.05	0.0003	1.06	1.04–1.08	<0.0001
Male gender	1.79	1.18–2.73	0.0062	2.89	1.81–4.59	<0.0001
Past and or daily ethanol intake >30 g/day	1.3	0.86–2.00	0.2			
Diabetes	1.44	0.95–2.19	0.084			
BMI (kg/m^2)	1.07	1.03–1.12	0.0008	1.06	1.021–1.11	0.0075
ALT (XUN)	0.98	0.9–1.07	0.73			
Bilirubin (μM/l L)	1	0.99–1.02	0.66			
Platelet count (10 000 units increase)	0.95	0.95–0.95	0.01	0.94	0.94–0.94	0.0024
Prothrombin activity (%)	1	0.98–1.02	0.79			
Albumin (g/L)	0.98	0.94–1.02	0.38			
AFP (ng/mL)	1	1.00–1.01	0.23			
HCV genotype 3	3.19	1.68–6.06	0.0004	3.54	1.84–6.81	0.0002
Grade of (>10%)	0.92	0.60–1.41	0.69			
Steatosis ≥30%)	1.32	0.73–2.39	0.36			

Median (q1–q3) for continuous variables. BMI, body mass index; ULN, upper normal classes 0 (<10%), 1 (10–30%) and 2 (>30%).

to potential confounding factors as prothrombin activity or alcohol intake. This leads to consider that HCV type 3 infection *per se* is responsible of our increased HCC prevalence.

Most of previous epidemiological studies that have investigated the impact of the HCV genotype on HCC occurrence in patients with established cirrhosis included few patients infected with a genotype 3 [23]. A previous study aimed at evaluating the impact of diabetes in patients with advanced fibrosis (30% with genotype 3) did not reported an impact of the genotype on HCC occurrence. However, this study included a high percentage of SVR (30%). But the authors did not specify the rate of SVR among patients infected with a genotype 3 nor reported the impact of genotype in the subgroup of patients who did not clear the virus [24]. In contrast to the study by Bruno *et al.* [25], in our study, patients with HCV genotype 2 and who did not achieved a SVR were as likely to develop HCC as patients with genotype 1 or 4. Difference in epidemiological distribution of genotype 2 patients can in part explain this difference. It should be note that in the study of Bruno *et al.*, only 22% of patients infected with a genotype 2 were older than 60 years *vs* 45% in their whole cohort and 50% in our study.

In our study, steatosis was more frequent in liver specimens of cirrhotic patients infected with a genotype 3 but its grade was not associated with HCC development. A case–control study based on liver explants of patients with established HCV cirrhosis reports a significant association between steatosis grade and the presence of HCC. But liver impairment was more severe in the group of patients transplanted without HCC [26]. Steatosis was also reported as associated with HCC occurrence in cohort patients with HCV including patients without severe fibrosis [27], but not in the series patients with advanced fibrosis and cirrhosis reported by Cadosa *et al.* [22]. As in the study of Lok *et al.*, we found an inverse relation between the degree of steatosis and the severity of the cirrhosis. Their study of paired biopsies of patients with HCV cirrhosis clearly shows that steatosis regresses during the transition from advanced fibrosis to cirrhosis and progression of portal hypertension [28]. On one hand, steatosis seems to be predictive of fibrosis progression and is observed in condition favouring HCC as metabolic syndrome or alcohol abuse. On the other hand, steatosis decreases with the progression of fibrosis and the severity of the cirrhosis, which is a main factor favouring HCC development. Nonetheless, the absence of predictive value of the degree of steatosis in patients with established cirrhosis (conversely to what has been suggested in patients with chronic hepatitis without cirrhosis) does not completely exclude that a long pre-existing period of lipid storage and exposure to lipid peroxidation has favoured the occurrence of subsequent HCC. As in the study of Chen *et al.*, we find a correlation between higher serum AFP level and liver steatosis [29].

An alternative explanation for linking genotype 3 infection with HCC occurrence is that mechanisms of steatogenesis such as increased SREBP or FAS activation could favour carcinogenesis by themselves, not directly through steatosis [11] or faster fibrosis progression because of steatosis.

Gene related to lipid metabolism as well as the liver content in fatty acids is modified in core-transfected transgenic mice that develop steatosis prior to HCC [30]. Further studies comparing the expression of genes involved in hepatic lipogenesis, level of lipid peroxidation and the composition of fatty acids of liver specimens of matched patients infected with a genotype 3 with those infected with others genotypes would provide important information on the mechanisms that underlie the relationship between infection with genotype 3, fat deposition and cancer. One limitation of our study is the retrospective design that did not allow the evaluation of possible confounding epidemiological factor associated with genotype 3 HCV and, particularly, cannabis consumption which have been previously reported as associated with liver steatosis [31]. Even if Adinolfi *et al.* showed that in patients with the same prevalence of drug use, the rate of liver steatosis was higher (75%) in patients infected with genotype 3, *vs* 22% in those with genotype 1a [17,32]. Further large prospective cohort including the cannabis consumption is needed to confirm our finding.

In conclusion, our study showed that in patients with HCV cirrhosis and persistent viral replication, infection with HCV genotype 3 is associated with an increased risk of developing HCC. This higher incidence seems independent from well-known risk factors and from the grade of steatosis although increased in these patients.

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