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TLL1 VARIANTS DO NOT PREDICT HEPATOCELLULAR CARCINOMA DEVELOPMENT IN HCV CIRRHOTIC PATIENTS TREATED WITH DIRECT-ACTING ANTIVIRALS

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Running title: TLL1 SNPs and HCC in DAA-treated HCV

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ABSTRACT

Tolloid like 1 gene (TLL1) variant *rs17047200* has been associated with hepatocellular carcinoma (HCC) in Japanese hepatitis C virus (HCV) patients with sustained virological response (SVR) to Interferon or direct-acting antivirals (DAA)-based regimens. We investigated if this holds true also in Caucasian cirrhotic patients cured by DAAs. Consecutive Caucasian HCV cirrhotics receiving DAA between December 2014-2016 in a single Center were enrolled. Cirrhosis was defined histologically (METAVIR F4) or by liver stiffness measurement (LSM >11.9 kPa). TLL1 *rs17047200* was analyzed by TaqMan SNP genotyping assay. 452 patients were enrolled: median age 63 (28-87) years, 58% males, 47% HCV-1b, LSM 19.1 (12.0-75.0) kPa, Fibrosis-4 (FIB-4) score 4.9 (0.3-46.0). 96% patients achieved an SVR. TLL1 genotype was AA in 329 (73%), AT/TT in 123 (27%) (MAF=0.14, HWE $p>0.05$). Patients' clinical features were similar across TLL1 genotypes. After 33 (3-47) months from DAA start, 31 patients developed HCC, 3-year estimated cumulative probability being 7.5% (95% CI 5-10%). Cumulative incidence of HCC was 9% in TLL1 AA vs. 7% in AT/TT patients ($p=0.55$). Male sex (HR 3.78, 95% CI 1.4-10.1, $p=0.008$), diabetes (HR 3.5, 95% CI 1.68-7.27, $p=0.001$) and FIB-4 (HR 1.09, 95% CI 1.03-1.14, $p=0.001$) were baseline independent predictors of HCC. Incidence of HCC was not influenced by TLL1 genotypes even when considering an additional group of 348 non-cirrhotic patients, being 2% in AA vs. 1% AT/TT patients ($p=0.58$). In a large cohort of Caucasian HCV cirrhotics treated with DAA, TLL1 variants do not predict HCC development.

KEYWORDS: TLL1, *rs17047200*, HCC, DAA, HCV, cirrhosis

1. INTRODUCTION

The achievement of a sustained virological response (SVR) to antiviral treatment for hepatitis C virus (HCV) has been shown to improve survival in patients with HCV-related cirrhosis, by reducing or preventing the occurrence of liver-related complications, such as hepatic decompensation and variceal bleeding [1,2]. On the other hand, persisting risk of hepatocellular carcinoma (HCC) in cirrhotic patients calls for maintaining long-life surveillance with 6-month-ultrasound (US) assessment [3,4]. Robust literature data have demonstrated that the incidence of HCC is reduced in cirrhotic patients cured through Interferon (IFN)-based regimens, and this seems to hold true also for those patients with an SVR to IFN-free treatments based on the use of direct-acting antivirals (DAA). However, the residual HCC risk in cirrhotic patients is estimated approximately 2% per year [5,6]. In this scenario, the possibility to identify HCC predictors has gained relevant importance, in order to develop individualized risk profiles that would allow for personalized surveillance strategies. Recently, a genome-wide study (GWAS) showed a strong association between toll-like receptor 1 gene (TLL1) variant *rs17047200* and HCC in IFN-cured HCV patients, and a Japanese study confirmed these results also in patients treated with DAA [7,8]. However, no data currently exist concerning TLL1 variants and risk of HCC in Caucasian patients with an SVR to DAA-based regimens. With this in mind, we aimed to evaluate the association between TLL1 variants and risk of HCC occurrence in a single-center large cohort of HCV cirrhotics treated with DAA.

2. METHODS

Consecutive Caucasian HCV cirrhotic patients treated with DAA between December 2014 and December 2016 were retrospectively enrolled in a single Hepatology center. Main exclusion criteria were HBV and HIV co-infection, previous history of HCC, active HCC or atypical/uncharacterized liver nodules at baseline (DAA start) (Supplementary Figure 1). Liver disease was staged either histologically (according to METAVIR score) or non-invasively (liver stiffness measurement [LSM]); LSM > 10 kPa and > 11.9 kPa for F3 and F4 fibrosis,

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respectively. Fibrosis-4 (FIB-4) score was also assessed. An SVR was defined as undetectable HCV-RNA 12 weeks after the end of treatment (Abbott-RT PCR, LLOD 12 IU/mL). HCC surveillance and diagnosis were performed according to international recommendations. Genotyping for TLL1 *rs17047200* was assessed with TaqMan single-nucleotide polymorphism genotyping allelic discrimination assay (Applied Biosystems, Foster City, CA). Genotypes were determined by the SDS software (v.2.2, StepOne Plus; Applied Biosystems). Prevalences of the TLL1 genotypes were reported by calculating the Minor allele frequency (MAF); moreover, consistency with the Hardy-Weinberg Equilibrium (HWE) was assessed.

Univariate and multivariable Cox proportional hazards regression analysis was used to assess the predictors of HCC development. Data handling and analysis were performed with Stata package (SAS Institute Inc., Cary, NC).

3. RESULTS

The study enrolled 452 cirrhotic patients: median age was 63 (28-87) years, 58% males, 47% HCV-1b, median ALT 74 (8-770) U/L, platelet count 115 (26-753) $\times 10^3$ /mL, alpha-fetoprotein (AFP) 9 (1-537) ng/mL, 20% with diabetes. Median LSM was 19.1 (12.0-75.0) kPa, FIB-4 score 4.9 (0.3-46.0), 87% Child-Pugh-Turcotte (CPT) score A, 34% with baseline esophageal or gastric varices (Supplementary Table 1). Overall, 96% patients achieved an SVR.

3.1 TLL1 genotype and patients' clinical features

TLL1 *rs17047200* genotype was AA in 329 (73%), AT in 116 (26%) and TT in 7 (1%) (MAF = 0.14, HWE $p > 0.05$). By comparing AA and AT/TT patients, there were no significant differences according to baseline clinical features, such as median age (63 years in both groups, $p = 0.49$), male sex (56% vs. 62%, $p = 0.29$), ALT

values (73 vs. 74 U/L, $p=0.36$), CPT score A (86% vs. 89%, $p=0.34$), or AFP values (9 vs. 10 ng/mL, $p=0.39$). Moreover, TLL1 genotype did not influence rates of SVR: 96% vs. 98% in AA vs. AT/TT patients, respectively ($p=0.57$).

3.2 Incidence and predictors of HCC

During 33 (3-47) months of follow-up from DAA start, 31 (6.9%) out of 452 patients developed de-novo HCC, with a 3-year cumulative probability of 7.5% (95% CI 5-10%). The 3-year cumulative incidence of HCC was 9% in TLL1 AA vs. 7% in AT/TT patients ($p=0.55$) (Figure 1). Male sex (HR 3.78, 95% CI 1.4-10.1, $p=0.008$), diabetes (HR 3.5, 95% CI 1.68-7.27, $p=0.001$) and FIB-4 (HR 1.09, 95% CI 1.03-1.14, $p=0.001$) were baseline independent predictors of HCC. Median HCC size at diagnosis was 20 (10-80) mm, single in 71%, BCLC stage 0-A in 87%, median AFP at HCC onset was 6 (2-9,240) ng/mL. Patients carrying the AA or AT/TT genotype were similar according to the main HCC features, such as nodule size (21 vs. 16 mm, $p=0.5$), number (single nodule in 81% vs. 50%, $p=0.1$), BCLC stage (0-A in 90% vs. 80%, $p=0.57$), and AFP values at HCC diagnosis (6 ng/mL in both groups, $p=1.0$). TLL1 genotypes were not associated with HCC onset even if HCC developed before SVR were excluded: the 3-year cumulative incidence was 7% in AT/TT vs. 4% in AA patients ($p=0.35$) (Supplementary Figure 2).

Following these results, we decided to investigate the relationship between TLL1 and HCC also in a non-cirrhotic cohort, i.e. in absence of advanced liver fibrosis as the main HCC risk factor. Therefore, we tested TLL1 in 348 patients staged F0-F3 who started DAA during the same timeframe of the original cirrhotic cohort. Demographic and clinical characteristics of the patients are shown in Supplementary Table 2: median age was 60 (21-88), 48% males, 45% HCV genotype 1b, median LSM 8.1 kPa (2.0-11.9), all without previous HCC history. TLL1 genotype was AA in 270 (78%), AT in 74 (21%) and TT in 4 (1%) (MAF 0.12, HWE $p>0.05$). During a median follow-up of 23 (5-42) months, HCC occurred in 3 (0.9%) patients, with a 3-year cumulative incidence of 2% (95% CI 1-5%). As for cirrhotic patients, incidence of HCC did not differ

according to TLL1 genotypes, being 2% in AA vs. 1% AT/TT patients ($p=0.58$). Of note, all three HCC developed in patients with F3 fibrosis: median size was 20 (12-38) mm, single in two cases, BCLC stage 0-A in all patients.

4. DISCUSSION

To the best of our knowledge, this is the first study evaluating the association between TLL1 and HCC in Caucasian HCV cirrhotic patients achieving an SVR following DAA. In this setting, we were not able to confirm the significant association between TLL1 and HCC reported by Matsuura and colleagues in IFN-treated and by Iio and coworkers in DAA-cured patients [7,8]. Indeed, in a single-center cohort of 452 consecutive patients, cumulative incidence of HCC after DAA did not differ according to TLL1 genotype. Moreover, TLL1 genotype did not influence HCC features at diagnosis. These results held true also when the effect of TLL1 was assessed in an additional cohort of 348 non-cirrhotic patients, thus reconfirming the lack of association between TLL1 and HCC also in patients with milder fibrosis. Several factors may potentially explain the differences between our study and the original Japanese ones. Firstly, the allele frequency difference among ethnic groups could reduce the *rs17047200* effect. Moreover, patients in the Matsuura study were followed-up for a longer time period than those from our Center (61 vs. 33 months). Finally, other co-factors affecting HCC risk (i.e. diabetes, alcohol) could differently contribute to HCC risk in the Japanese and Caucasian populations. Although the role of TLL1 protein has to be still elucidated, it seems to be involved in extracellular matrix assembly processes, and is presumed to enhance HCC development by altering hepatic fibrogenesis [7]. Since our study was retrospective, we were not able to confirm the association between TLL1 and fibrosis progression suggested by John et al [9], nor we could evaluate the correlation between TLL1 and fibrosis regression after SVR suggested by Huang and coworkers [10]. However, in our study, prevalence of the AT/TT genotypes did not differ across cirrhotic and non-cirrhotic patients (37% vs. 28%, respectively, $p=0.12$). Not surprisingly, both in our study and in the paper from Iio

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and colleagues, higher FIB-4 was independently associated with HCC [8], thus suggesting that more advanced liver fibrosis is a key risk factor for HCC, apart from genetics. Further reinforcing the predominant role of liver fibrosis stage as main HCC driver over genetics is the fact the all HCC observed in the non-cirrhotic population in our study occurred in F3 patients. Strength of our study is the single-center design, which granted for homogeneous enrolment criteria as well as availability of complete patients and HCC data. Conversely, the limited number of decompensated patients we included in the analysis potentially prevents us from observing more HCC events in a subset with higher tumour risk. In conclusion, in our large single-center cohort of HCV cirrhotics treated with DAA, TLL1 genotype was not associated with HCC development after viral cure.

FIGURE LEGEND

Figure 1. Cumulative probability of HCC according to TLL1 genotype during 33 (3-47) months follow-up in patients with cirrhosis

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SUPPLEMENTARY MATERIALS:

SUPPLEMENTARY TABLES

- Supplementary Table 1

- Supplementary Table 2

SUPPLEMENTARY FIGURES LEGENDS

Supplementary Figure 1. Study design and patient disposition

Supplementary Figure 2. Cumulative probabilities of HCC according to TLL1 genotypes when excluding HCC occurred before SVR12 timepoint

Supplementary Figure 3. Cumulative probabilities of HCC according to TLL1 genotypes in 348 non-cirrhotic HCV patients treated by DAA

Figure 1.

