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IL-28B *rs12979860* polymorphism affect the course of chronic hepatitis and the development of HCC in Egyptian patients with hepatitis C type 4

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ABSTRACT

Background: A single nucleotide polymorphism (SNP) in the interleukin 28B (IL28B) gene may alter the trajectory of hepatitis C virus (HCV) chronic infection. Several studies have sought to determine a link between IL28B *rs12979860* SNP and the development of HCV-related hepatocellular carcinoma (HCC), but with variable results, and consensus is awaited. We hypothesised that IL28B *rs12979860* SNP is linked to HCC in patients with HCV type 4.

Methods: IL28B genotyping of 300 patients with HCV-related fibrosis (n = 100), cirrhosis (n = 100) and HCC (n = 100) was carried out and the results were analysed to determine the association between the IL28B genotype and clinical outcome.

Results: In IL28B TT genotype carriers, the proportions of moderate/severe fibrosis, advanced cirrhosis (Child B-C) and HCC (50%, 84% and 60.2%, respectively) were higher ($p < 0.05$) than in CC/CT (4.3%, 46% and 23%, respectively). IL-28B SNP was linked significantly ($p < 0.05$) with cirrhosis progression and HCC advanced stages. Moreover, HCC advanced Child, Okuda and CLIP stages were associated with T allele carriage (73.9%, 82.6% and 78.3% *vs*. 44.2%, 50.6% and 46.8% in CC/CT). The percentage of large tumour size (> 3cm) increased ($p = 0.028$) in TT genotype carriers (81.8% *vs*.52.6% in CC/CT).

Conclusion: IL-28B *rs12979860* TT genotype is more prevalent in patients with advanced fibrosis, cirrhosis and HCC stages. Thus, it seems to be associated with poor outcomes in chronic HCV patients and to augment the risk of developing HCC.

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Introduction

More than 71 million people worldwide are estimated to have chronic hepatitis C (CHC) infection [1]. A significant number will develop cirrhosis and hepatocellular carcinoma (HCC), the latter being responsible for significant morbidity and mortality [2]. In patients with CHC, many factors – including alcohol intake, hepatitis B virus co-infection and male sex – can accelerate disease progression [3]. Host genetic factors have been suggested to be associated with the chronic phase, including HCC, and the development from acute HCV infection [4]. Moreover, variants of genes can determine the susceptibility to the development of HCC.

Single nucleotide polymorphisms (SNPs) are the most common form of human genetic polymorphism and are thought to be associated with susceptibility to cancer, population diversity and individual response to drug treatment [5]. The interleukin-28B (IL28B) *rs12979860* polymorphisms allelic variants may modify the treatment efficacy in CHC [6]. For instance, IL-18-137G/C (*rs187238*) SNP may be a factor that increases the risk of HCC [7]. Many studies have investigated the association between IL-28B *rs12979860* C/T SNP and the risk of liver cirrhosis and HCC development in various populations, although results are inconclusive and inconsistent [8]. For example, some studies reported that the T allele of IL-28B *rs12979860* appears to be more prevalent in patients with HCV-related liver cirrhosis and that this allele carriage seems to enhance the risk of the development of HCC [9]. However, others did not find any association between this SNP and HCC risk [10].

Previously, we reported that carriage of the IL-28B T allele increased liver cirrhosis severity; however, C allele coexistence with T allele reduces cirrhosis severity [11]. In this study, we hypothesised an association between IL-28B allelic distribution and the risk of HCC and a link with clinicopathological tumour features and common clinical staging systems.

Material and methods

Study population. Blood samples of 300 HCV-infected patients were collected for this study from Endemic Medicine Department, Cairo University Hospitals, Cairo, Egypt. They were equally divided into three groups (100 patients with liver fibrosis, 100 with liver cirrhosis and

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100 with HCC). An informed consent to participate in the study was obtained from each subject, in accordance with the Declaration of Helsinki and the local ethics committee of the hospital. Patients with chronic liver disease other than HCV infection wre excluded, such as infection with hepatitis B and/or human immunodeficiency virus. HCC patients were newly diagnosed, and tumours were previously untreated. The HCC diagnosis was confirmed either clinicopathologically or histopathologically, from combined examinations of ultrasonography and CT or biopsy specimens [12].

Staging systems. Fibrosis was coded according to the METAVIR [13] system: no fibrosis (F0), portal fibrosis (F1), portal fibrosis with rare septae (F2), portal fibrosis with many septae (F3) and cirrhosis (F4). Cirrhosis and HCC were classified according to Child-Turcotte-Pugh (CTP) [14] into early (A) and advanced (B-C) stages. The Okuda [15] staging system includes parameters associated with tumour stage (proportion of liver-involved area) and with liver functions (albumin, bilirubin and ascites). The CLIP score (7 points (0–6)) [16] was also used by integrating the alpha fetoprotein (AFP) level, CTP stages (A, B and C), tumour morphology (uninodular, multinodular and massive) and portal vein thrombosis (absence/presence). HCC patients were classified to: Okuda early (stage 1) and advanced (stages 2–3) stages and CLIP early (stage 0–1) and advanced (stage ≥ 2) stages. Hepatocellular carcinoma-AFP-routine test (HCC-ART) that includes routinely, noninvasive and simple measured markers increases the HCC screening reliability. HCC-ART = [age (years) × log AFP (UL^{-1}) × AST/ALT ratio × ALP ($U\ L^{-1}$)]/[Albumin (g^{-1})] [17,18].

Laboratory tests. All patients were tested HCV positive using ELISA (Biomedica, Sorin, Italy) and PCR (COBAS Ampliprep/COBAS TaqMan, Roche Diagnostics, Pleasanton, USA). Serum aspartate and alanine aminotransferases (AST and ALT), alkaline phosphatase (ALP), total bilirubin and albumin were measured using an automated biochemistry analyser (A15, Biosystem, Spain). The AFP level was estimated by chemiluminescence, with an Immulite (1000) AFP kit (Diagnostic Products Corporation; Los Angeles, CA, USA). Platelet count was performed using a KX-21 Sysmex automated haematology analyser (Sysmex Corporation, Kobe, Japan).

Molecular genetics In all patients, the genomic region encompassing the IL-28B *rs12979860* SNP was sequenced by PCR-based restriction fragment length polymorphism (RFLP). From whole blood samples, genomic DNA was extracted by a commercial kit (Roche Diagnostics GmbH, Mannheim, Germany). The PCR product was obtained with the forward primer 5′-GCGGAAGGAGCAGTTGCGCT-3′ and the reverse primer 5′-GGGCTTTGCTGGGGGAGTG-3′. In a total volume of 25 µL 1X PCR reaction buffer, 100–150 ng genomic DNA as a template, 2U/reaction of Taq DNA polymerase (Fermentas, Thermo Fisher Scientific, Boston, MA, USA) and 125 µmol each of deoxynucleotide triphosphates (dNTPs) were included for DNA amplification. The reaction mixture was subjected to cycles of initial denaturation at 94 °C for 5 min, 35 cycles including denaturation at 94 °C for 30 s, annealing at 65 °C for 30 s and elongation (at 72 °C for 30 s) and a final elongation (at 72 °C for 5 min) using a Veriti 96-well thermal cycler (Applied Biosystems, Carlsbad, CA, USA). In a total volume of 20 µL, 10 µL of the amplified product was digested with Bst U1 restriction endonuclease (5U) (New England Biolabs, Ipswich, MA, USA) at 60 °C overnight. The fragments digested were electrophoresed on 3% agarose gel along with a 100 bp ladder and visualised by a gel doc unit. After staining with ethidium bromide, fragments were 196 bp + 45 bp for C allele and 241 bp for T allele.

Statistical analysis Data are reported as mean with standard deviation (SD). ANOVA or Student t-test (continuous variables) and X^2 test or Fisher exact test (categorical variables) were used to identify differences. All tests were two-tailed, and significance was assessed at the 0.05 level. Bivariate Spearman's rank correlation coefficient measured the link between IL-28B SNP and the progression of liver fibrosis, cirrhosis and HCC stages. Analyses were performed by SPSS and GraphPad softwares.

Results

Demographic and diagnostic data, including age, gender, biochemical analysis, diameter and number of tumours, the presence of ascites, encephalopathy, enlarged spleen and portal thrombosis, genotyping at the polymorphic sites *rs12979860* of IL-28B are shown in Table 1.

IL-28B *rs12979860* genotyping showed that 208 (69.3%) of the 300 CHC patients were C allele carriers (CC and CT combined) and 92 (30.7%) were of TT genotype. Liver disease severity increased with the increase of TT genotype frequency: 12%, 30% and 50% in liver fibrosis, cirrhosis and HCC, respectively. In CHC patients with liver fibrosis, IL-28B *rs12979860* genotyping was present in 88 patients with C allele and in 12 with the TT genotype. Advanced fibrosis (F2-F3) was more frequent in patients with the TT genotype in C allele carriers. Early fibrosis (F0-F1) was more frequent in C allele carriers (Figure 1(a)). In cirrhotic patients, IL-28B *rs12979860* genotyping assessed 70 patients with C allele and 30 with the TT genotype. Similar to liver fibrosis, advanced cirrhosis (CTP B-C) was more frequent in patients with the TT genotype compared with C allele carriers (Figure 1(b)).

When HCC and non-HCC (fibrosis and cirrhosis combined) patients were compared, HCC development increased in patients with the TT genotype (Figure 2(a)). HCC in advanced CTP, Okuda and CLIP stages was more frequent in patients with the TT

Table 1. Clinical characteristics of patients.

Variable	Fibrosis	Cirrhosis	HCC	*P* value
Number	100	100	100	
Age (years)	39.2 (5.3)	49.4 (6.2)	54.3 (10.2)	< 0.001
Gender (male/female)	55/45	58/42	57/43	0.291
AST (U/L)	41 (9)	73 (8)	87 (16)	< 0.001
ALT (U/L)	51 (10)	61 (12)	68 (11)	0.023
ALP (U/L)	84 (13)	103 (26)	183 (38)	< 0.001
Albumin (g/L)	43 (10)	32 (5)	31 (5)	< 0.001
Platelet count ($\times 10^{-9}$/L)	200 (36)	124 (26)	115 (20)	< 0.001
Total bilirubin (μmol/L)	8 (1.5)	26 (8)	19 (6)	< 0.001
Log AFP (U/L)		1.35 ± 0.1	1.81 ± 0.15	< 0.001
Enlarged spleen (Yes/No)		2/98	4/96	
Portal vein thrombosis (Yes/No)			20/80	
*C allele carriers/TT	88/12	70/30	50/50	0.034

Abbreviations: AST = aspartate aminotransferase; ALT = alanine aminotransferase; ALP = alkaline phosphatase and AFP = α-fetoprotein. Continuous variables were expressed as mean (SD). *IL-28B *rs12979860* SNP. C allele carriers = CC combined with CT. Differences between independent groups were compared with the ANOVA test, whereas Pearson's chi-squared test (X^2) was used for nonparametric variables. Two HCC patients had encephalopathy and 29 had ascites.

genotype compared with C allele carriers (Figure 2(b–d)). Our previously developed HCC-ART score [17,18] significantly ($p < 0.0001$) increased in HCC patients with the TT genotype (11,778 ± 2367) than in C allele carriers (5577 ± 620). TT genotype was associated with large tumours (Figure 3(a)), but there was no association between *rs12979860* and the number of nodules or serum AFP (Figure 3(b–c)). Moreover, although weak, there was a significant link between IL-28B SNP and cirrhosis progression ($p = 0.030$) and the progression of CTP ($p = 0.049$), Okuda ($p = 0.033$), CLIP ($p = 0.018$) and HCC-ART ($p = 0.001$) HCC stages.

Discussion

HCV is one of the main causes of chronic liver disease worldwide [19]. In a previous study [11], we suggested that IL-28B *rs12979860* T allele affects the natural course of CHC type 4. Based on liver stiffness, a model for end-stage liver disease and elevated extracellular matrix proteins, this allele was associated with cirrhosis severity [11]. The present study extends our earlier work in that we report that the TT genotype frequency increased with liver disease severity and HCC development and that advanced fibrosis (F2-F3) and advanced cirrhosis (CTP B-C) were more frequent in patients with the TT genotype.

IFN-λ3 (encoded by *IL28B*) has a series of activities including anti-proliferative, antitumour and antiviral actions [20–25]. In neuro-endocrine BON1 tumour cells, IFN-λ1 and IFN-λ2 potently induced STAT signalling and anti-proliferative effects [21]. Moreover, in oesophageal cancer cell lines, IFN-λ1 determined growth suppression by the induction of G1 phase arrest or apoptosis [22]. When the B16 melanoma cells line, injected into mice, constitutively expressed IFN- λ2, the proliferation of these cells was abolished or retarded [23]. The proliferation of colon26 cancer cells, transduced with mouse IFN-λ, was markedly inhibited, and IFN-λ overexpression in hepatic cells increased the NK/NKT cells and enhanced their tumour-killing activity [24]. In the HCC transplantable hepatoma model, inoculated with BNL hepatoma cells expressing IFN-λ, IFN-λ possessed antitumour activity [25]. These findings and others suggest a link between the IL-28B *rs12979860* SNP and the occurrence of HCC.

HCC is the most advanced step in the course of CHC infection [26]. When HCC patients are compared with non-HCC (fibrosis and cirrhosis combined), HCC development increased in patients with the TT genotype compared with C allele carriers. If there is any association between IL28B SNP and the severity of HCV-induced liver disease, the most logical result should be

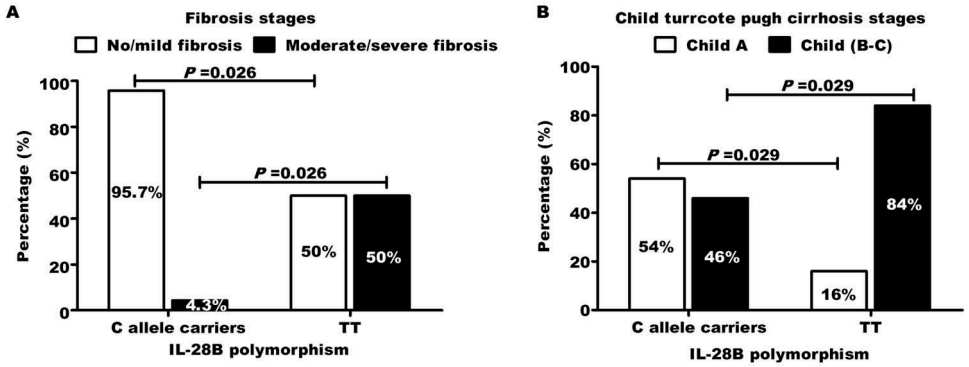

Figure 1. Percentage of advanced (a) fibrosis and (b) cirrhosis stages among IL28B SNP alleles. The TT genotype is significantly associated with moderate/severe fibrosis (F2-F3) and advanced cirrhosis (CTP B-C).

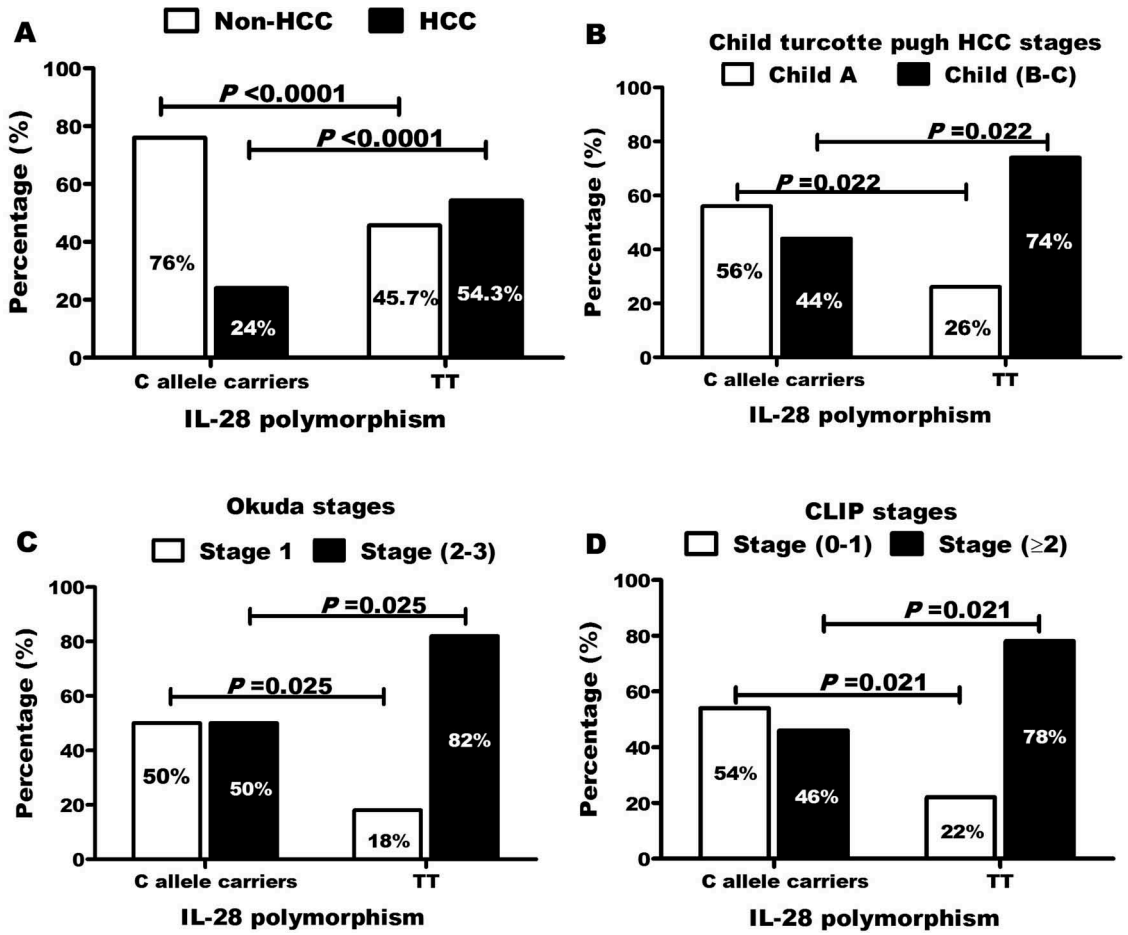

Figure 2. Association between IL28B *rs12979860* polymorphism and HCC development and severity. HCC is more frequent in IL28B TT genotype than C allele carriers. Non-HCC were fibrosis and cirrhosis combined. (a) HCC advanced, (b) Child–Turcotte–Pugh, (c) Okuda and (d) CLIP stages were more frequent in IL28B TT genotype than C allele carriers.

the presence of differences in IL28B genotype frequencies among different disease stages [10]. In this respect, we show that HCC in advanced CTP, Okuda and CLIP stages was more frequent in patients with the TT genotype compared with C allele carriers. IL28B TT genotype was associated with large tumours. Moreover, IL-28B SNP was linked significantly with cirrhosis progression and HCC advanced stages.

Our findings extend those of others. Fabris et al. found that the IL28B T allele is linked with the presence of HCC in liver-transplanted patients, and this allele was more frequent in patients with HCV-related HCC in comparison to non-HCV-infected ones [9]. When this analysis was extended to include patients who did not undergo transplantation, the relation between T allele and hepatic tumour was confirmed only in patients with viral-related cirrhosis [9]. Eurich et al. reported that HCC prevalence among explanted livers was significantly higher in patients with the TT genotype, suggesting a protective role of the C allele in HCC development [27]. El-Awady et al. reported that the sharp decrease in the CC genotype from healthy to CHC patients was associated with end-stage liver disease [28]. In a cohort of HBV-related HCC, carriers of the IL28B *rs12979860* T allele had a higher HCC risk compared with non-carriers [29].

Other studies have not found these links. Bochud et al. reported that IL28B SNP is not predictive of the HCC development in chronically HCV-infected patients [30], and Miura et al. reported that IL28B SNP was not related to HCC development. However, they acknowledged that this result was unexpected, because it is considered that IL28B SNP has a significant influence on the amino acid 70 residue of the HCV core that affects HCC development [31]. Another reason for this controversy is the HCV genotype. All patients of our study had HCV type 4, whereas other studies focused on other HCV genotypes, especially type 1. The link between IL28B SNP and the chronic course of HCV may be HCV genotype dependent [32]. Moreover, two meta-analyses, which included studies of two races (Caucasian and Asian), showed that there was a significant link between IL28B *rs12979860* TT genotype and HCC risk, particularly in patients with HCV-related HCC [33,34].

The strengths of the study include that it concerns the link between IL28B *rs12979860* SNP and HCC in HCV type 4. There are few data regarding the role of *IL28B* SNP in HCV-4 infection, and we report an association between IL28B genotypes and HCC CTP, Okuda and CLIP stages. In our previous study [11], C allele carriage protected from unfavourable clinical outcomes. The C allele coexistence with T allele

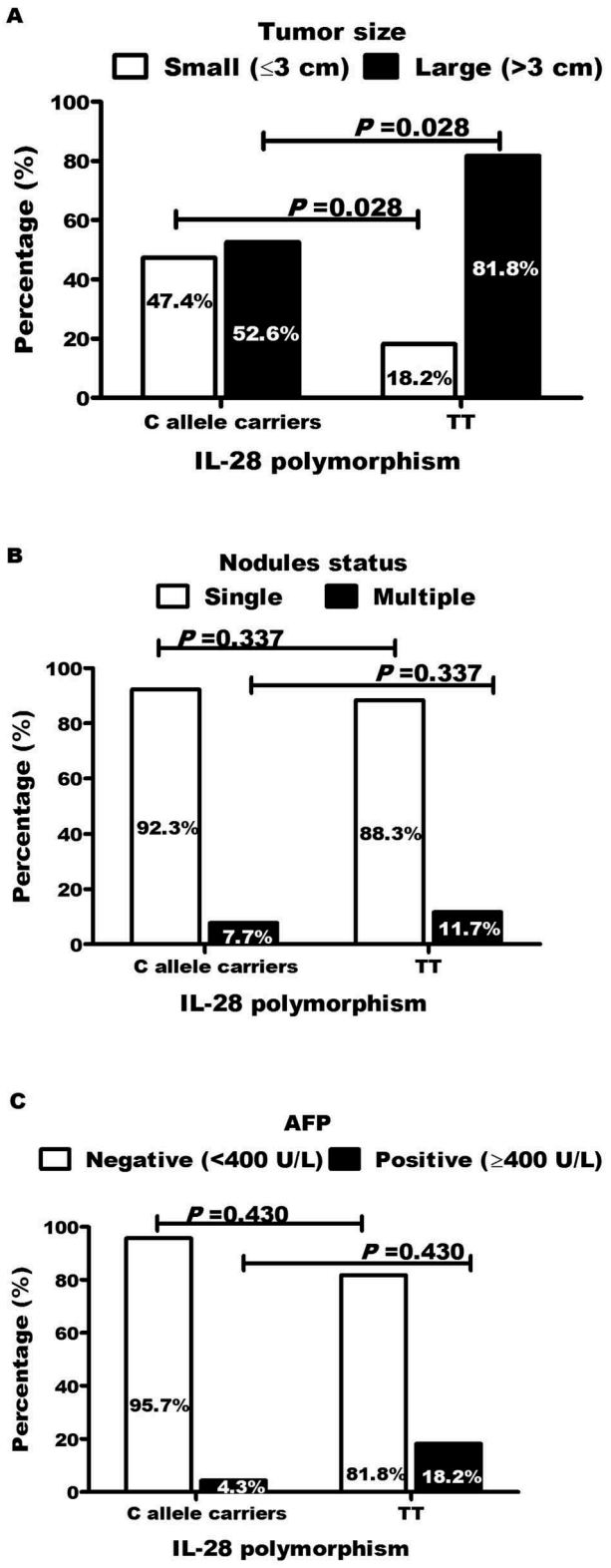

Figure 3. IL28B *rs12979860* polymorphism and some tumour features. The TT genotype is associated with (a) large tumours, but there is no association with (b) the number of nodules or (c) AFP serum levels.

reduced cirrhosis severity, as shown when comparing cirrhosis severity in CT carriers with the TT genotype. This is the reason for using the *rs12979860* (CC+ CT/TT) genetic model in this study. Other studies used the same model for IL28B *rs12979860* in evaluating the therapeutic response of patients with CHC-type 4 [35]. If confirmed, our results could have clinical consequences in the group of cirrhotic patients for whom early HCC detection might need to be focussed.

In conclusion, we found that the IL28B *rs12979860* T allele augments the severity of HCV-4-induced liver fibrosis, cirrhosis and HCC; nevertheless, its role cannot be excluded in HCC induced from different HCV genotypes. Carrying the T allele was associated with advanced HCC stages. Whether the major C allele plays a protective role against HCC development and whether the TT genotype is linked with tumour activity are worthy of further study. This work represents an advance in biomedical science because it highlights the role of IL28B *rs12979860* in HCV-induced HCC and the link between this SNP and HCC advanced stages.

Summary table

What is known about this topic:

- In CHC infection, IL28B *rs12979860* SNP allelic variants may modify the treatment efficacy.
- Carriage of IL-28B T allele increases liver cirrhosis severity, and C allele coexistence with T allele reduces severity.
- Results are inconclusive and inconsistent when studying the association between IL28B SNP and the risk of HCV-related HCC in variant populations.

What this work adds:

- IL28B *rs12979860* T allele augments the severity of HCV-4-induced HCC.
- Carrying T allele is associated with advanced HCC stages.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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