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Extracellular Matrix Proteins Substantiate IL-28B T allele Effect on Histological Outcome of Chronic Hepatitis C

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ABSTRACT

Introduction and aim. The correlation between interleukin-28B (IL-28B) polymorphisms and chronic hepatitis C (CHC) progression is debatable. Here, we aimed to evaluate the relation between IL-28B C/T genotypes and the development of cirrhotic liver. Extracellular matrix (ECM) proteins, FibroScan and model for end-stage liver disease (MELD) were used to substantiate the severity of liver disease. **Material and methods.** IL-28B *rs12979860*, liver stiffness and ECM proteins were assessed in 272 CHC patients. **Results.** Cirrhosis percentage increased to 10%, 52% and 96% with the increasing number of T alleles (CC, CT and TT, respectively). Also, elevated ECM proteins levels were correlated with the increasing number of T alleles. Interestingly, among cirrhotic patients, liver stiffness, MELD and ECM proteins were significantly ($P < 0.0001$) higher in patients with TT more than CT genotype. FibroScan, hyaluronic acid, Laminin, Collagen IV and the N-terminal pro-peptide of collagen type III have high accuracy to differentiate liver status in CC from TT genotype. Area under receiver-operating characteristic curve (95% CI) were 1.0 (1.0-1.0), 0.97 (0.96-1.0), 0.93 (0.85-1.0), 0.98 (0.97-1.0) and 0.93 (0.91-0.97), respectively. **Conclusion.** This study suggests that IL-28B T allele affects the natural course of CHC type 4 and also suggests that carriage of the IL-28B C allele protects from unfavorable clinical outcomes in CHC as coexistence of C allele with T allele reduced cirrhosis severity.

Key words. Interleukin-28B. Polymorphisms. Chronic hepatitis C. Fibrosis severity. Cirrhosis.

INTRODUCTION

Chronic infection with hepatitis C virus is a global health problem that affects millions of people worldwide. In CHC patients, some host's genetics provide some explanation for HCV different outcomes and influence liver fibrosis, particularly modifiers in genes controlling the inflammatory and immune response pathways.[1] In this context, *rs12979860* single-nucleotide polymorphisms (SNP) of IL-28B being the strongest host factor associated with viral clearance.[1,2] Limited and controversial data suggests that IL-28B SNP relate to the severity of hepatic histology.[3] However, the *rs12979860* good response C allele may be associated with greater hepatic inflammation, higher alanine aminotransferase levels and increased risk of worse clinical outcomes,[2,3] other studies have not found this association.[4] Another study reported that CC genotype had lower mean Ishak fibrosis scores, compared with other genotypes.[2] Furthermore, other studies found that the T allele affects the severity of liver fibrosis and had a mean staging score higher than other genotypes.[5,6] In HCV type 1 infection, Kitson, *et al*. reported that IL28B *rs12979860* T allele appears to be related to higher prevalence of advanced fibrosis stages.[3] Also, none of the previous studies concerned the association between the IL-28B SNP and signs of fibrosis severity.

Liver parenchymal cells replace and regenerate the apoptotic or necrotic cells after acute liver injury. During

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chronic injury, liver regeneration fails, and hepatocytes are substituted with abundant ECM, including fibrillar collagen.[7] Thus, alterations in the quantity, composition and reorganization of ECM proteins are one of the main processes of liver fibrogenesis. In advanced fibrosis stages, the liver contains approximately 6 times more ECM components than normal level.[7] Therefore, measuring such ECM parameters in CC, CT, and TT IL-28B genotypes may provide us a unique opportunity to study the conundrum and identify the genotype that predicts liver fibrosis progression. Distribution of IL-28B genotypes is varied in various races[8] and limited data investigating this distribution among CHC Egyptian patients with overwhelming majority of cases having hepatitis C virus (HCV) genotype 4.

Based on these premises, we aimed to assess the allelic and genotypic frequencies of IL-28B *rs12979860* in HCV infected Egyptian individuals. Also, to investigate serum levels of ECM proteins, including HA, laminin, collagen type IV and PIIINP in different IL-28B *rs12979860* genotypes as well as its association with liver disease progression and elevated values of MELD score.[9]

MATERIALS AND METHODS

Study population

Two hundred seventy two CHC Egyptian patients infected with HCV genotype 4 were randomly recruited from Endemic Medicine Department, Cairo University Hospitals, Cairo, Egypt. To be included, patients had to be aged 18 or more, infected with HCV and not infected with HBV or HIV and not received antiviral therapy. Study protocol was conducted in accordance with the ethical principles and guidance of the Helsinki Declaration. All patients provided written consent to participate in the study.

Stiffness measurement

According to the previously described technique,[10] transient elastography was carried out by using FibroScan™ (Echosens, Paris, France), a dedicated medical device which provides a quantifiable estimate of liver stiffness in kilopascal (kPa).

Laboratory tests

All patients were tested positive for anti-HCV antibodies (Biomedica, Sorin, Italy) and HCV-RNA (COBAS Ampliprep/ COBAS TaqMan, Roche Diagnostics, Pleasanton, USA). Liver function tests were measured on an automated biochemistry analyzer (A15, Biosystem, Spain). Complete blood count was performed using KX-21 Sysmex automated hematology analyzer (Sysmex Corporation, Kobe, Japan). Alpha fetoprotein (AFP) level was estimated by chemiluminescence, with Immulite (1000) AFP kit (Diagnostic Products Corporation; Los Angeles, CA, USA).

Levels of liver fibrosis markers in serum

Serum levels of HA, laminin, collagen type IV and PIIINP were determined using a double-antibody sandwich ELISA according to the manufacturer's instructions (Shanghai Sunred Biological Technology Co., Ltd, Shanghai, China). Assay range of human HA ELISA kit (catalogue No. 201-12-1375) is 4-600 ng/mL, human laminin ELISA kit (catalogue No. 201-12-1562) is 1.5-400 ng/mL, human collagen type IV ELISA kit (catalogue No. 201-12-1381) is 0.2-40 μg/mL, and human PIIINP ELISA kit (catalogue No. 201-12-1359) is 0.5-90 ng/mL.

Molecular detection of interleukin-28B genotypes

Using restriction fragment length polymorphism (RFLP) from whole genomic DNA, genotyping for the IL-28B *rs12979860* was determined. After genomic DNA isolation from whole blood using a commercial kit (Roche Diagnostics GmbH, Mannheim, Germany), the isolated DNA was electrophoresed on 1% agarose gels for quality check and quantified using nanodrop for purity. The PCR was performed using the following primer pairs: 5'-GCGGAAGGAGCAGTTGCGCT-3' and 5'-GGGCTTTGCTGGGGGAGTG-3'. As a template, 100-150 ng genomic DNA was included in each reaction in 25 μL having 1X PCR reaction buffer. Each forward and reverse primer was added to a final concentration 10 pmol/ reaction. Taq DNA polymerase (Fermentas, Thermo Fisher Scientific, Boston, MA, USA), 2U/reaction, was used for DNA amplification, with 125 μmol each of deoxynucleotide triphosphates (dNTPs). The reaction mixture was subjected to PCR: initial denaturation at 94 $^{\circ}$C for 5 minutes, 35 cycles, including denaturation at 94 $^{\circ}$C for 30 seconds, annealing at 65 $^{\circ}$C for 30 seconds, and elongation at 72 $^{\circ}$C for 30 sec, and final elongation at 72 $^{\circ}$C for 5 min using Veriti 96-well thermal cycler (Applied Biosystems, Carlsbad, CA, USA). For RFLP analysis, 10 μL of amplified product were digested with 5 U of Bst U1 restriction endonuclease (New England Biolabs, Ipswich, MA, USA) in a total volume of 20 μL at 60 $^{\circ}$C for overnight. The restriction digested DNA was electrophoresed on 3% agarose gel along with a 100 bp ladder and visualized by a gel doc unit (Biorad, Hercules, CA, USA). The digested fragment was 196 bp and 45 bp for CC genotype; 241 bp, 196 bp, and 45 bp for CT; and 241 bp for TT genotype.

Statistical analysis

Statistical analyses were performed by SPSS software v.15.0 (SPSS Inc., Chica go, IL) and GraphPad Prism package; v.5.0 (GraphPad Software, San Diego, CA). Data were expressed and reported as mean ± standard error of the mean (SEM). Differences in continuous variables were assessed using ANOVA or Student t-test and χ^2 test or Fisher exact test for categorical variables. Pearson correlation test was used to investigate the relation between two variables among each group. All tests were two-tailed and statistical significance assessed at the 0.05 level. The diagnostic performances value was assessed by area under receiver-operating characteristic curve (AUC) analysis. The cut-off points were selected according to the point on the curve closest to the (0, 1) point (the minimal $(1\text{-sensitivity})^2+(1\text{-specificity})^2$).[11]

RESULTS

IL-28B genotype distribution among CHC Egyptian patients

The clinical data of patients included in this study showed that patients with IL-28B genotypes CC, CT and TT were not significantly different ($P > 0.05$) with respect to any assessed variables including ALT, AST, albumin, total bilirubin, alpha fetoprotein, platelet count and HCV-RNA (data not shown). The RFLP findings revealed that IL-28B *rs12979860* CT genotype is the commonest genotype among Egyptian patients infected with HCV genotype 4. The CC genotype constituted ≈ 18% (48/272) of the studied sample, while the TT genotype constituted ≈ 9% (24/272) and the heterozygous genotype CT ≈ 73% (199/272).

IL-28B polymorphism and severity of fibrosis according to FibroScan

The cirrhosis percentage was significantly higher in patients with the IL-28B TT alleles than in those with CT or CC alleles (96%, 52% and 10%, respectively; Figure 1a). There was no significant correlation ($P > 0.05$) between IL-28B polymorphism and liver function parameters. On the contrary, FibroScan values (kPa) gave a strong positive correlation ($r = 0.6$; $P < 0.0001$) with IL28B polymorphism and there was stepwise increase in these values from CC to TT genotypes (Figure 1B).

ECM proteins confirm that IL-28B T allele is associated with advanced disease

Elevated ECM proteins levels in patients serum were associated with the presence of IL-28B T allele (Table 1; Figure 2A-D). Similar to FibroScan, HA ($r = 0.5$), laminin ($r = 0.5$), collagen IV ($r = 0.4$) and PIIINP ($r = 0.4$) serum levels showed significant ($P < 0.0001$) positive associations with IL28B polymorphism. In comparison with CC genotype, IL-28B *rs12979860* T allele had a significant 2.4-fold increase (in case of CT) and 4.7-fold increase (in case of TT) in Fibroscan (kPa). The same was true for ECM proteins serum levels (Figure 2E). This addresses the robustness of FibroScan findings. Mean MELD score in TT genotype (9.6 ± 0.73) in patients with liver cirrhosis was highly increased in comparison with CT genotype (3.9 ± 0.41).

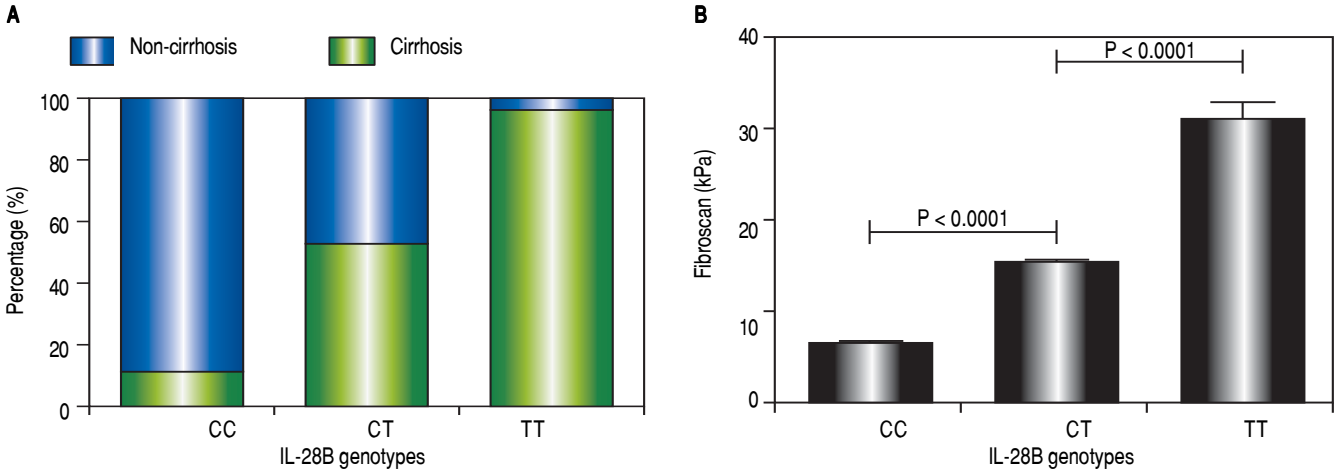

Figure 1. *Distribution of IL-28B* rs12979860 *genotypes in CHC patients and FibroScan values according to IL-28B* rs12979860 *SNP.* ***A.*** *Distribution of cirrhotic and non-cirrhotic subjects among IL-28B* rs12979860 *genotypes.* ***B.*** *The increase in FibroScan values (kPa) showed the difference between CC, CT and TT genotypes.*

Table 1. Distribution of different fibrosis markers in IL-28B CC, CT and TT genotypes.

Fibrosis marker	IL-28B genotypes ‡			*P* value
	CC	CT	TT	
FibroScan (kPa) †	6.6 ± 0.4	15.6 ± 0.6	31.1 ± 2.3	0.0001
Hyaluronic acid (ng/mL) †	74.6 ± 5.2	147.8 ± 8.2	419.4 ± 95.2	0.0001
Laminin (ng/mL) †	60.6 ± 3.2	106.5 ± 4.6	282.1 ± 58.1	0.0001
Collagen IV (µg/mL) †	6.1 ± 0.4	11.8 ± 0.9	30.1 ± 6.2	0.0001
PIIINP § (ng/mL) †	13.3 ± 0.7	25.6 ± 1.7	55.4 ± 10.4	0.0001

Variables were expressed as †mean ± SEM. ‡IL-28B rs12979860 single-nucleotide polymorphism. § PIIINP = the N-terminal pro-peptide of collagen type III. P > 0.05 is considered not significant, P < 0.05 considered significant.

IL-28B genotypes differentiation using fibrosis markers

The discriminatory power of fibrosis markers was evaluated based on the area under the ROC curve (Table 2). AUC values for FibroScan HA, laminin, collagen IV and PIIINP serum levels to differentiate CC genotype from the other IL-28B genotypes combined (CT and TT) were 0.91, 0.85, 0.84, 0.82 and 0.82, respectively. These values rose to 1.0, 0.97, 0.93, 0.98 and 0.93, respectively, when comparing CC to TT genotype (Figure 3).

DISCUSSION

In Egypt, the frequencies of the IL-28B *rs12979860* SNP were scarcely studied in HCV type 4 infected patients,[12,13] also with varied results. Herein, the scrutiny of a large cohort (272) of randomly selected HCV infected Egyptian patients revealed that the heterozygous genotype CT is the most common (73%) which is concordant, but in different percentages, with Pasha, *et al*. and Youssef, *et al*.[12,13] but not with Shaker and Sadik.[14] Moreover, this result was consistent with studies performed in countries other than Egypt. In Italy, Faileti, *et al*. reported that individuals with CT genotype are the most common patients infected with HCV genotype 4.[5]

In CHC patients, some studies have shown an association between CC genotype and more advanced fibrosis or cirrhosis.[2,3] Others have shown an association between TT genotype and more advanced fibrosis or cirrhosis.[5,15] While, other investigators reported no association of IL-28B SNP with fibrosis. Approximately, all studies using FibroScan to uncover the relation between IL-28B genetic variants and liver fibrosis are in the context of HCV genotype 1, 2 or 3.[16] In this study, FibroScan of infected patients with HCV genotype 4 revealed that the percentage of cirrhosis increased with the increasing number of T alleles as it was 10%, 52% and 96% in CC, CT and TT genotypes, respectively. Also, among cirrhotic patients, MELD score was elevated in TT genotype in comparison to CT genotype.

If IL-28B *rs12979860* SNP truly affects response to interferon therapy and changes the natural history of CHC, patients with the unfavourable alleles (T) should be over-represented in patients with end-stage liver disease (cirrhosis).[15] Fabris, *et al*., used Ishak staging score to assess liver fibrosis and cirrhosis and they found that patients with CHC-related cirrhosis carried more frequently the TT genotype in comparison to HBV-related cirrhosis or mild hepatitis C.[15] Also, IL-28B *rs12979860* CT/TT genotypes were associated with numerous markers of liver disease severity such as γ-glutamyl transferase and higher grades of steatosis.[17] Among liver transplant recipients, recipient IL-28B *rs12979860* SNP was significantly predictive of fibrosis stage, with TT genotype being associated with more rapid fibrosis.[18] In addition, some other studies reported that IL-28B T allele is frequently found in advanced fibrosis stages according to different scoring systems.[2,3,5,6]

Other finding of this study, concerns the demonstration of a strict association between IL-28B *rs12979860* T allele and elevation levels of HA, laminin, collagen IV and PIIINP in patients serum. Liver cirrhosis is associated with swathes of dense ECM rich in elastin fibrillar collagens and other matrix proteins. Indeed, elevation of these proteins is used as a pathological benchmark for liver disease severity.[19] HA levels increase with the progression of liver fibrosis to cirrhosis and HA levels correlate with clinical severity in the cirrhotic patients.[20] In cirrhotic liver, there are increases in fibrillar collagen, laminin and other proteoglycans compared with normal liver.[19] Natural killer (NK) cells can limit liver fibrosis by killing hepatic stellate cell-derived myofibroblasts, which represent the main source of ECM.[21] In IL-28B *rs12979860* T allele carriers, the function of these cells was depressed and induction of other innate immunity genes was also low.[22] Also, IL-28B T allele was associated with high hepatic miR-122 expression. As detected by FibroScan and histology, this miR-122 negatively correlates with advanced hepatic fibrosis.[23,24]

Liver cirrhosis is a state of an acquired immune deficiency and all host defense systems are compromised, e.g.

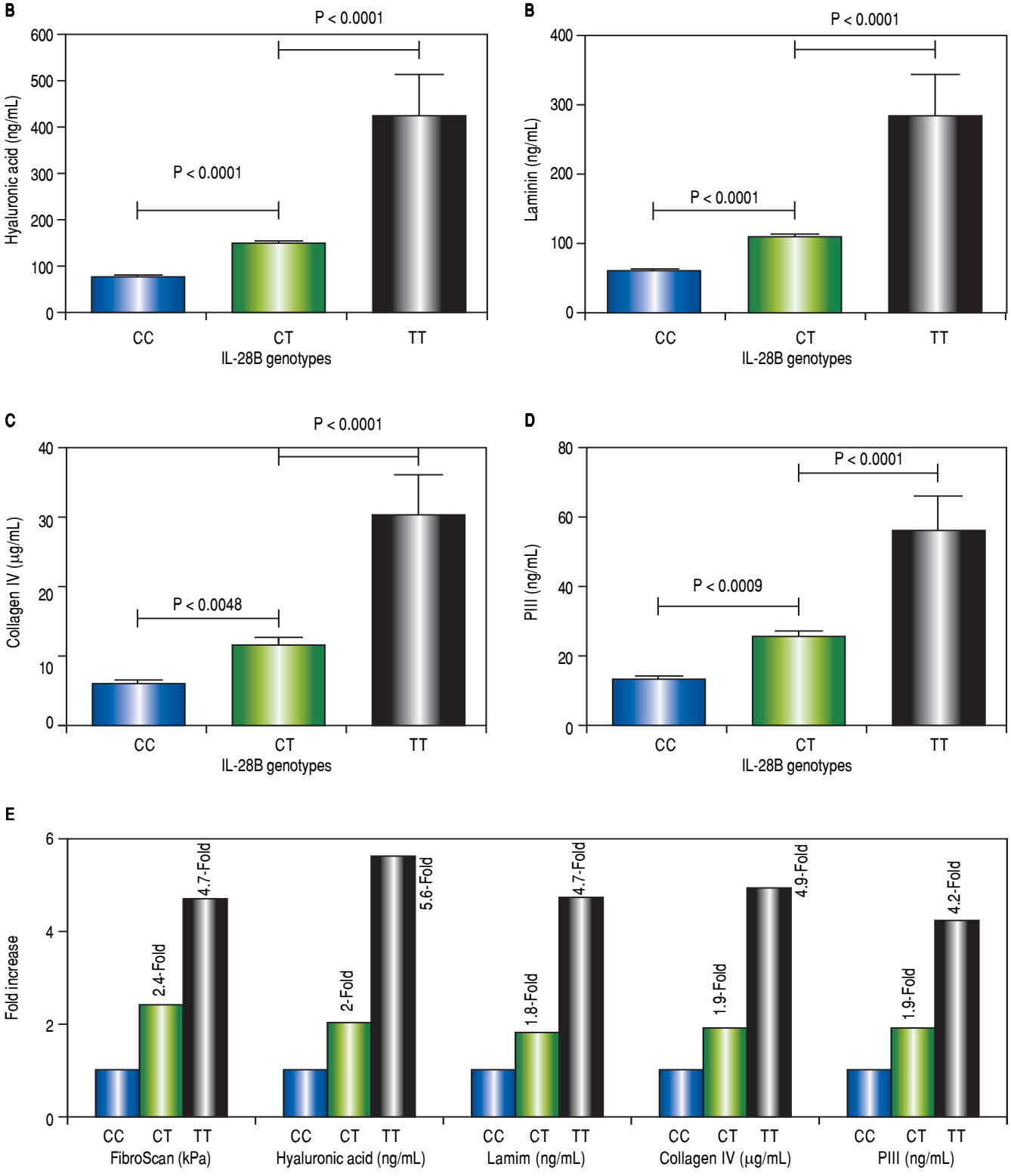

Figure 2. *Extracellular matrix proteins in patients with different IL-28B* rs12979860 *genotypes. EC|M circulating levels in patients serum* ***(A)*** *hyaluronic acid (ng/mL),* ***(B)*** *laminin (ng/mL),* ***(C)*** *collagen type IV and* ***(D)*** *N-terminal pro-peptide of collagen type III (PIIINP) (ng/mL) were increased with an increasing number of T alleles.* ***(E)*** *Data represent fold increase of FibroScan and extracellular matrix proteins relative to IL-28B* rs12979860 *SNP in patients with liver diseases.*

the acute phase response and neutrocyte, macrophage and lymphocyte functions.[25] In contrast to T allele, our results support data demonstrating that IL-28B C allele is linked with a better response to interferon-based antiviral treatment in CHC patients[26] and to a higher probability to virus clearance during the CHC natural history.[27] As de la Fuente, *et al*., suggested that IL-28B *rs12979860* SNP influences the natural course in HCV genotype 1,[28] our findings suggested that IL-28B *rs12979860* SNP affect also natural course of HCV genotype 4 and T allele increases the risk

Table 2. Performance of different liver fibrosis markers for differentiating different IL-28B genotypes.

Index	Cutoff	CC *vs.* CT/TT			
		AUC	95% CI	Sn%	Sp%
FibroScan (kPa)	8.5	0.91	(0.867-0.965)	83	87
Hyaluronic acid (ng/mL)	84	0.85	(0.804-0.919)	74	78
Laminin (ng/mL)	72	0.84	(0.788-0.926)	75	78
Collagen IV (μg/mL)	6.5	0.82	(0.766-0.894)	72	72
PIIINP (ng/mL)	14.5	0.82	(0.780-0.905)	78	78

PIIINP: the N-terminal pro-peptide of collagen type III. AUC: Area under receiver-operating characteristic curve for differentiating between different IL-28B genotypes. 95% C: 95% Confidence Interval. Sn: sensitivity. Sp: specificity.

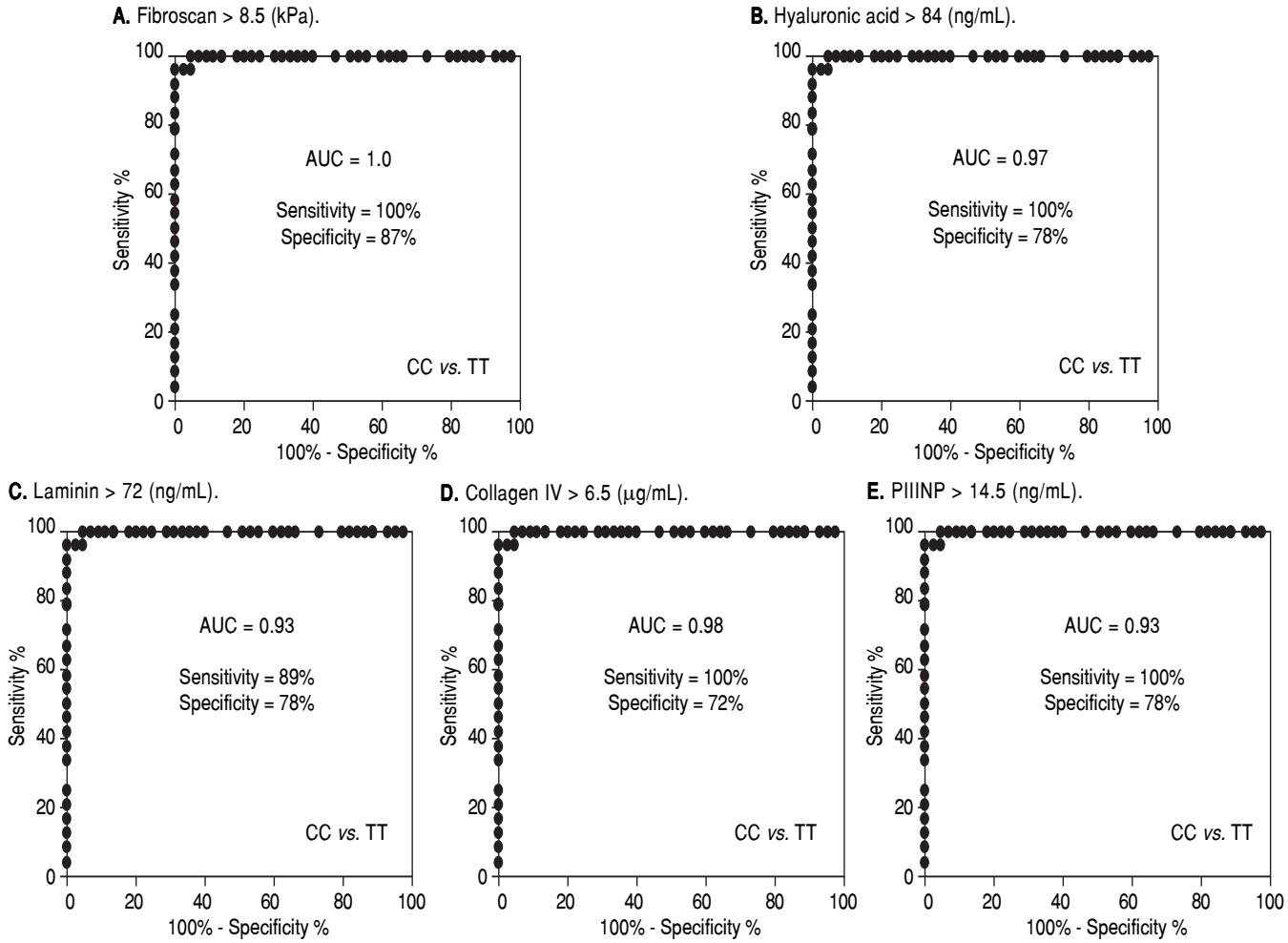

Figure 3. *Area under receiver-operating characteristic curve (AUC) of* ***(A)*** *Fibroscan (at 8.5 kPa),* ***(B)*** *Hyaluronic acid (at 84 ng/mL),* ***(C)*** *Laminin (at 72 ng/mL),* ***(D)*** *Collagen IV (at 6.5 μg/mL) and (****E****) PIIINP (at 14.5 ng/mL) for separating patients with IL-28B CC from patients with IL-28B TT genotype.*

of cirrhosis progression and need for liver transplant. In formal logic terms and from the obvious inferences, this study gives a good deduction that carriage of the IL-28B C allele protects from unfavorable outcomes in CHC.

Because of the highest predictive sustained virologic response associated with *rs12979860* CC genotype,[26] another interesting novel finding of this study concerns the differentiation of favorable (CC) from unfavorable (TT) genotypes using FibroScan and ECM proteins. AUC values for FibroScan HA, laminin, collagen IV and PIIINP serum levels to differentiate CC from other IL-28B genotypes were 0.91, 0.85, 0.84, 0.82 and 0.82, respectively which rise to 1.0, 0.97, 0.93, 0.98 and 0.93, respectively, when comparing CC to TT genotype only. So, these fibrosis indices are good options for routine diagnostic testing of liver condition in IL-28B genotypes.

The importance of this work stems from the following: This work concerns HCV genotype 4, as there is limited data about the relation between IL-28B *rs12979860* SNP and the severity of disease. Also, the relation between hepatic fibrosis and IL-28B *rs12979860* SNP was demonstrated not only using FibroScan but also the elevation of ECM proteins levels in patient's serum. This study raise the theoretical possibility that IL-28B *rs12979860* SNP may influences future HCV treatment regimen since T allele would increase the risk of end stage progression and need for liver transplantation. While, C allele may be not in need of liver transplantation owing to slower disease progression beside high rates of spontaneous viral clearance. This study shed light on the use of FibroScan and ECM proteins as good diagnostic options for liver disease severity in IL-28B genotypes.

This study was limited by its retrospective nature. Further multicenter prospective studies involving a greater number of patients are warranted. Any protective association between CC genotype and a lower risk of advanced liver disease warrants further evaluation. Also, other studies were recommended to evaluate this association in diverse subtypes of genotype 4 and other HCV genotypes.

In conclusion, this study suggests that IL-28B T allele affects the natural course of CHC type 4 and also suggests that carriage of the IL-28B C allele protects from unfavorable clinical outcomes in CHC as coexistence of C allele with T allele reduced cirrhosis severity.

ABBREVIATIONS

- **AFP:** Alpha fetoprotein.
- **AUC:** area under receiver-operating characteristic curve.
- **CHC:** chronic hepatitis C.
- **dNTPs:** deoxynucleotide triphosphates.
- **ECM:** extracellular matrix.
- **HCV:** hepatitis C virus.
- **IL-28B:** Interleukin 28B.
- **MELD:** model for end-stage liver disease.
- **NK:** Natural killer.
- **PIIINP:** N-terminal pro-peptide of collagen type III.
- **RFLP:** restriction fragment length polymorphism.
- **SEM:** standard error of the mean.
- **SNP:** single-nucleotide polymorphisms.

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CONFLICTS OF INTEREST

None.

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