TM6SF2 RS58542926 IS LINKED TO THROMBOCYTOPENIA-RELATED ADVANCED HEPATIC FIBROSIS IN EGYPTIAN PATIENTS WITH CHRONIC HEPATITIS C

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Thrombocytopenia in chronic hepatitis C (CHC) patients is a serious complication, which hinders starting and continuation of several invasive diagnoses and treatment protocols. This problem is multifactorial; therefore, it is important to explore host factors linked to it. Interestingly while we were investigating the association between rs58542926 polymorphism in TM6SF2 gene and both hepatic & extra hepatic changes, we found a significant association between TM6SF2 risk allele and thrombocytopenia in Egyptian CHC patients. There are 351 participants in this study. We measured gene expression of IL-6 and TNF-α in peripheral blood mononuclear cells (PBMCs) and plasma thrombopoietin (TPO) level in 20 CHC patients with advanced fibrosis. The minor allele was linked to elevated thrombocytopenia (TCP), and lower TPO levels. These findings raise concerns regarding the effect of this polymorphism on different diagnostic techniques and the outcomes of hepatitis C virus (HCV) treatment. In conclusion, we demonstrated for the first time that the occurrence of the minor allele of the rs58542926 polymorphism is associated not only with hepatic but also with extrahepatic (thrombocytopenia) alterations in CHC Egyptian patients, allowing for the use of this variant as a pharmacogenetic marker to recognize CHC patients who have a high possibility for developing late stages of liver fibrosis and extrahepatic complications.

Key words: TM6SF2; rs58542926; hepatitis C, thrombocytopenia

INTRODUCTION

HCV is a pervasive virus affecting more than 184 million around the world. It is the causing agent for about 50 % of death due to viral hepatitis which is the seventh major cause of death globally; therefore, the United Nations incorporated the plan to fight viral hepatitis

into its 2030 Agenda for Sustainable Development[1-3]. Egypt shows one of the highest prevalence ratios of about 14.7 % in 2009 and 10.0 % in 2015 with 40,000 deaths per year, ultimately higher than global levels[1]. Despite of the progress in the Egyptian national HCV treatment program and strategy for HCV control, present evidence shows continuous HCV spreading in Egypt, with greater prevalence rates compared with other nations[1]. Many patients (70-80%) fail to get rid of the virus spontaneously during the acute stage and progress to chronic infections[4]. Chronic Hepatitis C (CHC) viral infection is accompanied by both hepatic- and extrahepatic complications; therefore, mortality rates double in HCV carriers compared to healthy individuals[5]. Liver cirrhosis and hepatocellular carcinoma (HCC) are the most unpleasant-expected hepatic consequences of CHC infections. However, the highest mortality rates from HCV infections have been correlated to progressive hepatic fibrosis[2]. Fibrosis is a structural damage to the liver tissue that ranges from mild changes limited to the portal and peripheral areas to more severe changes that end up with cirrhosis in a time frame of approximately 20 years. Fibrosis is believed to be a result of many etiologies other than CHC, including obesity, inflammation, alcohol intake, and chronic viral infections. These multiple etiological factors suggest that genetic factors may also have a significant effect on the pathogenesis of chronic liver disease[6].

Additionally, there are many extrahepatic serious problems that are prevalent among CHC candidates such as thrombocytopenia (TCP). The prevalence of TCP among CHC patients is ranging from 0.16 % up to 76 % with a higher prevalence in patients with advanced liver fibrosis and cirrhosis[7]. The major obstacle affects the management of CHC populations with TCP is the complexity in starting and continuation of interferon (INF) based anti-viral treatment[8]. Even with recent advances in the eradication of HCV using direct-acting antivirals (DAAs), still some patients with specific variants are more susceptible to liver fibrosis secondary to CHC infection[9,10]. Moreover, TCP still should take a particular consideration, particularly in advanced hepatic disease because of excessive bleeding with TCP can cause several serious complications which may hamper the inception and permanence of various invasive diagnostic and treatment protocols[7&8]. TCP in CHC patients is multifactorial; therefore, it is necessary to know the underlying pathophysiological mechanism fostering it for good TCP management, especially the host factor or genetics-based one[8&11].

The transmembrane 6 superfamily 2 (*TM6SF2*) polymorphism is located at locus 19p13.3-p12 and was identified by an exome-wide association study[12]. The *TM6SF2* non-synonymous polymorphism rs58542926 was linked to non-alcoholic fatty liver disease (NAFLD)[12,13], higher prevalence of fibrosis and alcohol-related cirrhosis[14], triglyceride secretion[15], and myocardial infarction[16]. Additionally, it has been demonstrated that this variant decreases secretion of very low-density lipoprotein (vLDL) in vitro and causes fat to accumulate in the liver[12]. As a result, it was assumed that there is a link between rs58542926 variant in the *TM6SF2* gene and liver fibrosis in CHC patients. In the beginning, the role of the rs58542926 variant was controversial in the condition of CHC, until an Australian large-scale functional study showed a considerable correlation of this polymorphism with hepatic fibrosis in European patients with CHC[17], which was followed by a meta-analysis that supported the role of this variant in histological changes in CHC infection, where it showed a robust association between the *TM6SF2* variant and fibrosis development[18]. Therefore, the current study aims for the first time to explore the role of the rs58542926 variant and its association with both hepatic and extrahepatic alterations (e.g., TCP) in Egyptian CHC patients.

PATIENTS AND METHODS

Patient cohort

For gaining representative results, the study included 351 subjects (250 with CHC, and 101 healthy control subjects) from Al-Rajhi Liver Center, Assiut University Hospital, Assiut, Egypt, from 2018 to 2020. The inclusion criteria taken into consideration for selecting CHC subjects included CHC patients who underwent a FibroScan® and FibroTest® with fibrosis level scoring prior to starting the antiviral treatment. Patients who tested positive

using standard tests for other liver illnesses were excluded. The control group included healthy individuals with no history of chronic liver diseases. Ethical approval was obtained from the research ethics committee in the Faculty of Pharmacy, Minia University. Each participant gave a written informed consent and got information regarding the disease's nature and the diagnostic techniques involved.

Clinical and laboratory assessment

Whole blood samples (10 ml from each patient) and data collection as well as liver fibrosis staging were done at the same time. Viral load, serum bilirubin, serum albumin, alanine transaminase (ALT), Aspartate transaminase (AST), international normalized ratio (INR), and platelets count were evaluated using commercially available kits.

Staging of liver fibrosis

Both FibroScan®, a non-invasive test for liver fibrosis assessment, and FibroTest®, a highly sensitive serum biomarker test for evaluating liver fibrosis, were used for staging fibrosis to avoid the use of invasive techniques like taking biopsies[16]. Fibrosis was staged according to liver stiffness measuring by FibroScan®. Fibrosis was scored on a scale: stage F0-F1: < 7 Kpa, F2: ≥ 7 Kpa, F3: ≥ 9.5 Kpa (severe fibrosis), and F4: ≥ 12 Kpa (cirrhosis)[19]. A total of 73 patients out of 250 patients were categorized as F0-F1, 50 patients were categorized as F2, 26 patients as F3, and the remaining 101 patients were categorized as F4.

Genotyping

Genotyping for *TM6SF2* rs58542926 (n=351) was performed utilizing the TaqMan SNP genotyping allelic discrimination technique (Applied Biosystems, Foster City, CA) as in the producer's guidelines. All genotyping was concealed from clinical variables.

Determination of TPO level

Solid phase sandwich enzyme-linked immunosorbent assay (ELISA) (Invitrogen, USA) was used to measure thrombopoietin concentration in the plasma of 20 CHC patients with known *TM6SF2* rs58542926 genotype.

Separation of Peripheral blood mononuclear cells (PBMCs)

Ficoll Paque Plus (GE Healthcare) was used in a density gradient centrifugation procedure to isolate peripheral blood mononuclear cells (PBMCs).

mRNA gene expression

RNA extraction. Isolation of total RNA from cells was performed by the Tissue Total RNA purification (Qiagen miRNeasy Mini Kit as per manufacturer's guidelines). Reverse transcription of cDNA from total RNA was done using High-Capacity cDNA Reverse Transcription Kit (ThermoFisher).

Quantitative Real-time PCR (RT-qPCR).

SYBR green and primers for each gene were used in RT-qPCR which was performed by Applied Biosystems, Foster City, CA, USA, and the results were then normalized to the level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The values of CT used for the measurement of expression were calibrated to those of GAPDH ($\Delta CT = CT$ (GAPDH) – CT (target) and finally represented as $2-\Delta CT$. Primers sequence used in the experiment are mentioned in **Table (1)**

Table 1: Primer sequences for human GAPDH, TNF-α, and IL-1B, used for qRT-PCR analysis.

Gene	Primer sequence
GAPDH	Forward;5-GACTAACCCTGCGCTCCTG-3 Reverse;5-GCCCAATACGACCAAATCAG-3
IL-1β	Forward;5-ACAGATGAAGTGCTCCTTCCA-3 Reverse;5-GTCGGAGATTCGTAGCTGGAT-3
TNF-α	Forward;5-CAGGGACCTCTCTCTAATCA-3 Reverse;5- GTAATAAAGGGATTGGGGCA-3

Data analysis

The data were statistically analyzed using SPSS for windows version 16 (IBM Corp., Armonk, NY, USA) and GraphPad Prism version 8.0. (Graph pad software San Diego, USA). The normality of the data was assessed using the Shapiro-wilk Test of Normality. Using Mann-Whitney U test or Student's t-test, continuous data were compared and shown as means ± standard deviation (SD) or median and range. Using chi-squared ($\chi 2$), categorical variables were compared and represented as a percentage. The risk factors of severe hepatic fibrosis were determined by regression analysis. To be considered statistically significant, P should be less than 0.05.

RESULTS AND DISCUSSION

Results
Genotype distribution

The Frequency of *TM6SF2* rs58542926 minor allele (MAF) in the healthy group and CHC subjects showed almost similar values (10% and 8% respectively) (MAF = 0.06) (P = 0.7) **(Table 2).**

Table 2 shows no significant deviation from the Hardy-Weinberg equilibrium for healthy control and CHC patients (P= 0.7). Chi-square test was used to find P value.

When the distribution of the major and minor alleles in males and females of each group was compared, it was found that the percentage of females carrying the minor alleles was higher, however, there was no significant difference (P = 0.893).

The rs58542926 genotype is associated with TCP in CHC patients.

Comparison of the metabolic profile of CT/TT group against CC subjects showed that patients with the minor allele had significantly lower platelet count (thrombocytopenia), higher serum bilirubin, higher INR ratios, and lower serum albumin. The mean values of ALT, AST, and BMI did not significantly differ in the minor allele cohort (CT/TT) and the major allele one as shown in **Table 3**. HCV viral load did not differ significantly between patients with the *TM6SF2* rs58542926 (TT/CT) genotype and those with the CC genotype, despite the lower mean value for viral load in the former group.

Table 2: Distribution of rs58542926 and Hardy-Weinberg equilibrium.

rs58542926	CHC (Egyptian patients) (n=250)	Healthy controls (Egyptian population) (n=101)
CC	230 (92%)	91(90%)
CT	18 (7%)	9 (9%)
TT	2 (1%)	1 (1%)

Table 3: Characteristics of Egyptian CHC patients according to genotype of rs58542926.

Variables	TM6SF2 rs58542926 Genotype		P value
	CC (n=230)	CT/TT(n=20)	
Age	55.6±13.6 (19-87)	56.1±12.2 (29-84)	0.893
Gender (M/F) %	132/98(57.4/42.6%)	11/9 (55/45%)	0.836
BMI (Kg/m^2)	27.9 ± 6.2	28.6± 5.9	0.783
Serum bilirubin (mg/dL)	0.8 (0.2-3.9)	1.1(0.4-6.95)	0.014*
Serum albumin (g/dL)	3.6 ± 1	3.1 ± 1	0.044*
Platelet (x10^9/L)	190.3 ± 91.9	135.7 ± 73.2	0.01*
ALT (IU/L)	37 (3-259)	34 (13-146)	0.375
AST (IU/L)	40 (9-320)	44.5 (19-191)	0.274
INR	1.2 ± 0.4	1.5 ± 0.5	0.008*
HCV viral load	8.8X10^5 (5.4X10^3-3.8 X 10^7)	6.4 X 10^5 (4.9X10^4-1.2X10^6)	0.328

Association between rs58542926 and fibrosis stage in CHC subjects

After evaluation of the fibrosis stage with FibroTest and FibroScan, we next analyzed the association of the rs58542926 genotype with hepatic fibrosis stage. Interestingly, as shown in **Fig. 1**, the minor allele significantly associated with severe fibrosis stages, where 70% of subjects had staging of F3-F4, compared to the major allele which has 65% of subjects showed fibrosis stages of F0-F2 ($P = 0.005$). This association was confirmed by univariate and multivariate analysis **(Table 4).**
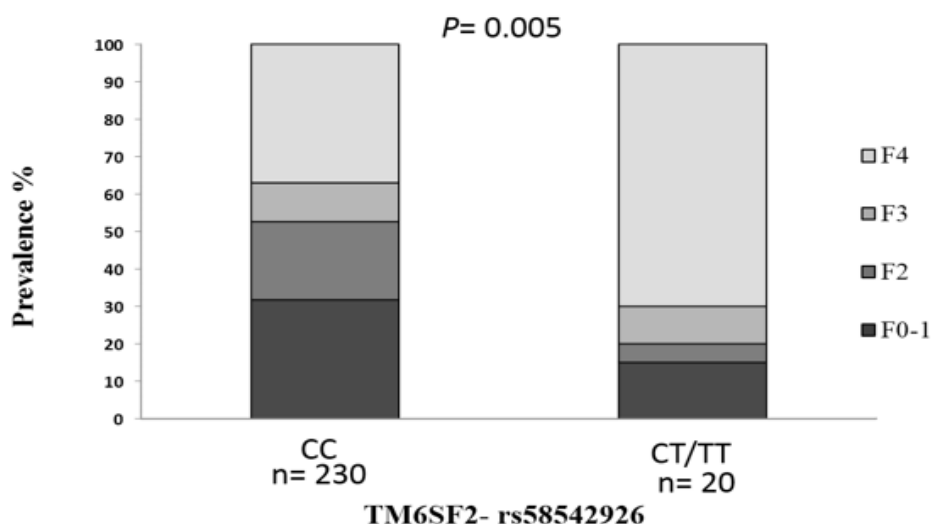

Fig. 1: Association of rs58542926 genotype to fibrosis stage in the Egyptian patients (n=250). *P*-values are univariate and provided for the dominant model of inheritance **.**

Table 4: Predictors of severe hepatic fibrosis (> F2).

	Univariate analysis		Multivariate analysis	
	Odds ratio (95% CI)	***P***	**Odds ratio (95% CI)**	***P***
Age	1.062 (1.038 - 1.086)	**< 0.001**	1.083 (1.031 - 1.136)	**0.001**
Gender	0.653 (0.394 - 1.081)	0.097	NA*	NA*
AST	1.037 (1.024 - 1.05)	**< 0.001**	1.034 (1.015 - 1.054)	**< 0.001**
ALT	1.006 (0.999 - 1.013)	0.115	NA*	NA*
Serum Bilirubin	1.208 (1.046 - 1.394)	**0.01**	1.016 (0.892 - 1.156)	0.816
Serum albumin	0.153 (0.095 - 0.246)	**< 0.001**	0.335 (0.148 - 0.762)	**0.009**
INR	1.778 (1.472 - 2.146)	**< 0.001**	1.081 (0.831 - 1.407)	0.561
PLT	0.973 (0.967 - 0.980)	**< 0.001**	0.982 (0.974 - 0.991)	**< 0.001**
PCR	1 (0 - 1.01)	0.942	NA*	NA*
TM6SF2	6.401 (1.826 - 22.441)	**0.004**	22.776 (2.068 - 250.797)	**0.011**
Thrombopoietin	0.979 (0.964 - 0.995)	**0.009**	NA*	NA*

* NA: not available.

Link between rs58542926 and plasma thrombopoietin level in CHC patients.

After observing the significant association of the minor allele with thrombocytopenia and advanced fibrosis level, it was interesting to explore whether this low platelet count can be regarded as a decrease in thrombopoietin levels, the primary regulator of platelet production. Measurement of plasma thrombopoietin concentration in 20 CHC subjects, with advanced hepatic fibrosis, revealed a significant association between rs58542926 minor allele and low plasma thrombopoietin level as shown in **Fig. 2** ($P = 0.0003$).

Expression level of inflammatory mediators' genes in PBMCs

To investigate the role of rs58542926 in the inflammation process as a predisposing process for fibrosis, we measured the levels of mRNA expression for TNF-α and IL-6. There was no significant association between the minor allele (T) and expression level of the measured inflammatory mediators. $P = 0.27$ and > 0.9 as represented in **Fig. 3**.
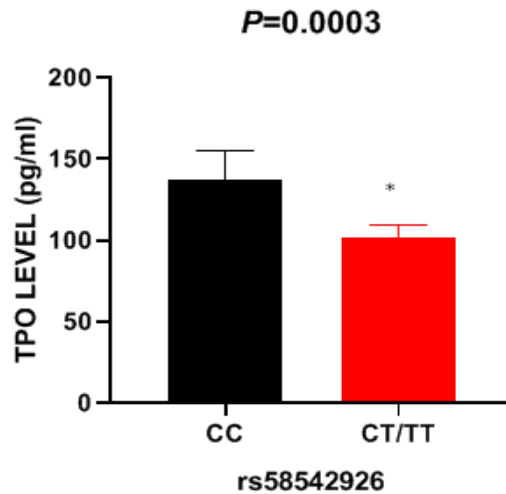

Fig. 2: Link between rs58542926 genotype and plasma TPO level in CHC patients with severe liver fibrosis (n=20). *P*-values are univariate and provided for the dominant model of inheritance.

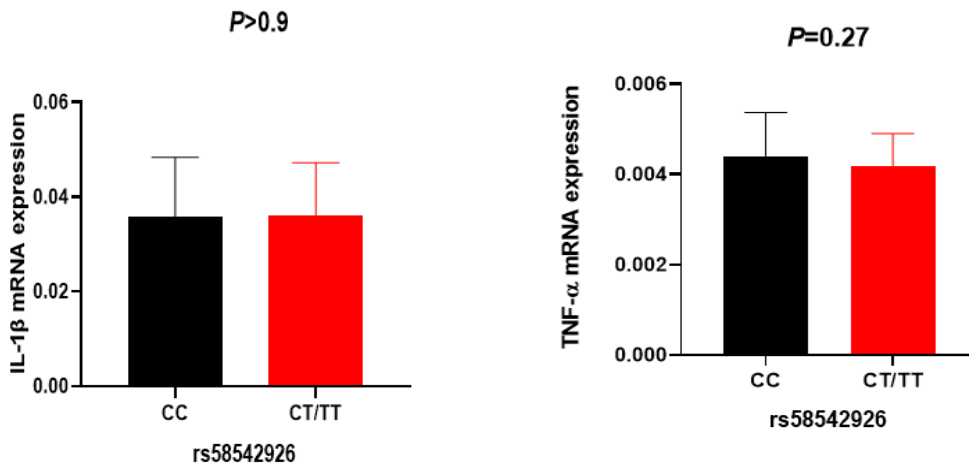**Fig. 3:** Gene expression of (A) IL-6 and (B) TNF-α and their association with rs58542926.

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Discussion

The current study explored the link of the *TM6SF2* rs58542926 to hepatic fibrosis level and extrahepatic complications (e.g., TCP) in Egyptian CHC patients. Based on our findings, it is evident that the *TM6SF2* rs58542926 enhances the development of both hepatic and extrahepatic alterations (e.g., TCP). The rs58542926 minor allele was linked to advanced hepatic fibrosis level but not inflammation as reported before in a different cohorts[17,18] and we also found an association with thrombocytopenia in an Egyptian cohort of 250 patients with CHC viral infection.

The most important hepatic alterations which occur due to CHC infection are liver cirrhosis, hepatocellular carcinoma (HCC), and end-stage liver disease, where the progression from fibrosis to cirrhosis can take ten to thirty years[20]. In addition, CHC patients with chronic HCV infection also suffer from several extra-hepatic pathologies that range from stroke and myocardial infarction to diabetes and autoimmune responses like rheumatoid arthritis[21]. Thrombocytopenia is a major concern in CHC patients, affecting almost 45 % of them[22]. This decrease in platelet count can induce bleeding manifestations, which strongly can influence the beginning and duration of antiviral treatment in the corresponding cases[22]. Thrombocytopenia can be observed in the majority of HCV groups with sever fibrosis and/or cirrhosis, compared to the non-cirrhotic[23,24]. Thrombocytopenia can limit not only diagnostic but also therapeutic procedures and treatments[25]. Moreover, it increases the threats of dangerous complications, particularly excessive bleeding[7]. The pathogenesis of thrombocytopenia in patients with CHC has been linked to a number of etiologies, such as increased platelet destruction either due to autoimmune reactions or platelet sequestration as a consequence of splenomegaly[25]. Additionally, HCV-induced bone marrow suppression, reduced TPO synthesis due to fibrosis, and treatment side effects are reasons that could contribute to CHC-associated thrombocytopenia[2,25]. Therefore, further research is recommended to clarify the underlying genetic causes behind the pathogenesis of chronic liver disease-associated thrombocytopenia.

Human genetic variants are gaining much interest in the last decade for their role in chronic liver disease- severity and its associated thrombocytopenia. Interestingly, a previous study showed that the minor allele of rs11697186 (located in the *DDRGK1* gene) was significantly associated with the decrease in platelet count that was happened during the treatment of HCV infection with peg-interferon[11].

In the current study, we found that the minor allele (T allele) was significantly associated with the occurrence of thrombocytopenia which matches the previous researches reporting this extrahepatic manifestation in CHC patients, especially who have severe liver fibrosis[23,24]. It is important to note that the observed thrombocytopenia in this work was linked to lower TPO plasma levels. These results are consistent with data from other studies that found that production of TPO was negatively correlated to the severity of liver fibrosis[26]. One possible explanation for the association between the *TM6SF2* minor allele and low plasma TPO level is the correlation of this variant with advanced-hepatic fibrosis level. As fibrosis advances to cirrhosis, the liver shrinks and loses its ability to synthesize TPO, resulting in inappropriately low levels of TPO[27]. Another explanation is the link between *TM6SF2* genotype, triglyceride, and TPO levels. In a previous study, the T allele of rs58542926 was significantly associated with low plasma triglyceride concentrations in CHC patients[15,17]. Triglyceride level was previously found to be a significant positive correlation with TPO level[28]

Conclusion

To sum up, our study shed for the first-time light on the association of the minor allele of rs58542926 with low serum TPO levels and with the fibrosis stage in Egyptian CHC patients and links the minor allele to the severe fibrosis stages in such cohort without any significant effect on inflammatory mediators as well as hazard extrahepatic alteration (e.g., thrombocytopenia). This introduces the minor variant of *TM6SF2* rs58542926 as a pharmacogenetic diagnosis tool to predict progression of hepatic and extrahepatic alterations in Egyptian cohort with CHC.

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نشرة العلوم الصيدليـــة
جامعة أسيوط

المتغير rs58542926 في جين عبر الغشاء ٦ من أفراد العائلة الفائقة ٢ مرتبط بقلة الصفائح الدموية في مرضي التليف الكبدي الشديد المصريين الناتج عن الإلتهاب الكبدي الفيروسي سي المزمن

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⁵ قسم الميكروبيولوجي والمناعة ، كلية الصيدلة ، جامعة المنيا ، المنيا ، ٦١٥١٩ ، جمهورية مصر العربية

⁶ قسم العقاقير والكيمياء الصيدلية، كلية الصيدلة ، جامعة طيبة ، المدينة المنورة ، المملكة العربية السعودية

⁷ قسم العقاقير والكيمياء الصيدلية، كلية الصيدلة ، جامعة الأزهر ، ١١٨٨٤،القاهرة ، جمهورية مصر العربية

⁸ قسم الكيمياء الحيوية، كلية الصيدلة ، جامعة الأزهر ، أسيوط ، ٧١٥٢٤ ، جمهورية مصر العربية

قلة الصفائح الدموية في مرضى الالتهاب الكبدي الفيروسي سي المزمن هي من المضـاعفات الخطيرة، خاصة في المرضى الذين يعانون من تليف الكبد الشديد ، مما يعيق بدء واستمرار العديد من التشخيصات وبروتوكولات العلاج. هذه المشكلة متعددة العوامل. لذلك، من المهم استكشـاف العوامـل الجينية المرتبطة بها. من المثير للاهتمام أنه أثناء بحثنا في العلاقة بين تعدد الأشكال (rs58542926) في جين عبر الغشاء ٦ من أفراد العائلة الفائقة ٢ (TM6SF2) والتغيرات الكبدية والغير كبدية ، وجدنا ارتباطًا مهمًا بين المتغير الخطر(T) في هذا الجين ونقص الصفيحات في مرضى الالتهاب الكبدي الفيروسي سي المزمن المصريين. هناك ٣٥١ مشاركًا في هذه الدراسة: ٢٥٠ منهم من حالات مرضى الالتهاب الكبدي الفيروسي سي المزمن بمستويات مختلفة من التليف (F0-F4) و ١٠١ متطوعًا سليمًا. تم تحديد النمط الجيني rs58542926 في جميع العينات. لفهم الآليات الوظيفيـة لتـأثير متغيـر TM6SF2 ، قمنا بقياس التعبير الجيني لـــ IL-6 و TNF-α فـي PBMCs ومستوى ثرومبوبويتين البلازما في ٢٠ من مرضى CHC المصابين بالتليف المتقدم. تم ربط المتغير(T) بتليف الكبد الشـديد، وارتفاع قلة الصفيحات ، وانخفاض مستويات الثرومبوبويتين. تثير هذه النتائج مخاوف بشأن تأثير تعدد الأشكال في هذا الجين على تقنيات التشخيص المختلفة ونتائج علاج التهاب الكبد الوبائي. في الختـام، أوضحنا لأول مرة أن وجود المتغير (T) لـ rs58542926 يرتبط بالتغيرات الكبدية والغير الكبدية

(قلة الصفيحات) في مرضى الالتهاب الكبدي الفيروسي سي المزمن المصريين ، مما يسمح باستخدام هذا المتغير كعلامة فارماكوجينية للتعرف على مرضى الالتهاب الكبدي الفيروسي سي المزمن الذين لديهم احتمالية عالية لتطوير المراحل المتأخرة من تليف الكبد والمضاعفات خارج الكبد.