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BRIEF ARTICLE

Increased fibrosis progression rates in hepatitis C patients carrying the prothrombin G20210A mutation

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Abstract

AIM: To examine whether hepatitis C virus (HCV)-infected patients who carry hypercoagulable mutations suffer from increased rates of liver fibrosis.

METHODS: We analyzed DNA samples of 168 HCV patients for three common hypercoagulable gene mutations: prothrombin 20210 (PT20210), factor V Leiden (FV Leiden) and methylene tetrahydrofolate reductase (MTHFR). The patients were consecutively recruited as part of the prospective "Fibroscore Study" in France. The effect of the various mutations on the rate of fibrosis was analyzed statistically and was correlated with epidemiological, clinical and biochemical data such as grade and stage of liver biopsies, patients' risk factors for liver cirrhosis, and timing of infection.

RESULTS: Fifty two of the patients were categorized as "fast fibrosers" and 116 as "slow fibrosers"; 13% of the "fast fibrosers" carried the PT20210 mutation as compared with 5.5% of the "slow fibrosers", with an odds ratio of 4.76 (P = 0.033; 95% CI: 1.13-19.99) for "fast" liver fibrosis. Carriage of MTHFR or FV Leiden mutations was not associated with enhanced liver fibrosis.

CONCLUSION: Carriage of the PT20210 mutation is related to an increased rate of liver fibrosis in HCV patients.

Key words: Hepatitis C virus; Liver fibrosis; Hypercoagulation; Prothrombin 20210

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INTRODUCTION

Cirrhosis is a major cause of morbidity and mortality in patients who suffer from chronic hepatitis C virus (HCV) infection. Up to 24% of patients will develop cirrhosis during their lifetime[1]. Various characteristics, such as male gender, older age at infection, alcohol consumption, obesity and concurrent hepatitis B or human immunodeficiency virus (HIV) infection enhance the rate of liver fibrosis[2,3]. Unfortunately, it is still largely impossible to predict who is more prone to fibrosis, and, thus, careful follow-up or treatment is required for most patients.

Hypercoagulable states have been hypothesized to play a role in organ fibrosis. In various inflammatory states, such as those of the lung or kidney, thrombosis and fibrin formation result in organ injury. It has been recently proposed that hypercoagulable states may be an additional contributing factor to liver fibrosis through several mechanisms, such as thrombotic events in small venous blood vessels in the liver and stimulation of hepatic stellate cells by thrombin[4]. Moreover, increased fibrin deposition has been demonstrated in animal models of liver fibrosis[5]. These observations have been strengthened by a study conducted by Anstee *et al*[6], which explored a mouse model of liver fibrosis and demonstrated that the extent of fibrosis was much higher in mice carrying the factor V Leiden (FV Leiden) mutation.

Primary hypercoagulable states, such as FV Leiden and prothrombin 20210 (PT20210), result from mutations in genes that encode proteins of the coagulation cascade[7]. FV Leiden results from a G1691A single nucleotide polymorphism gene mutation that leads to an amino acid substitution of arginine for glutamine at position 506 of the protein, which is one of the cleavage sites of activated protein C (APC). The mutated protein is more resistant to APC cleavage, and, as a result, the negative feedback on the coagulation cascade is impaired. Thus, the FV Leiden mutation is responsible for venous thromboembolisms at a high prevalence of 1%-8.5%[8]. Another common mutation involves the elevation of plasma prothrombin levels due to a G→A transition in nucleotide 20210 in the prothrombin gene. The prevalence of heterozygosity for this mutation among Caucasian populations is 1%-6%[9]. A third common mutation that results in hypercoagulation is a genetic variant of the *methylene tetrahydrofolate reductase* (*MTHFR*) gene, which leads to an elevation in homocysteine levels and an increased risk of venous and arterial thrombosis. Epidemiological data from humans have shown that APC resistance resulting from FV Leiden heterozygosity is related to an increased rate of fibrosis in HCV patients[4,10], as opposed to carriage of the PT20210 mutation, which has not been found to be a contributing factor to liver cirrhosis[10]. Hyperhomocysteinemia and MTHFR C677T mutations have been found to play roles in liver steatosis in HCV patients and, thus, indirectly play a role in the progression of liver fibrosis[11].

In this work, we examined whether a mutation in one of these genes contributes to accelerated liver fibrosis in French HCV patients.

MATERIALS AND METHODS

Patients

In this retrospective study, we analyzed data that were collected from HCV-infected patients. The first 168 consecutive patients were included from the "Fibroscore Study", which was a French national, multicenter, prospective, and cross-sectional study of Caucasian patients that was performed by researchers who are well known for their specific expertise in HCV in five centers in the southeast region of France, including Saint-Joseph Hospital and La Conception Hospital (Marseille), Archet Hospital (Nice), Hyeres Hospital and the Arnault Tzanck Institute (St. Laurent du Var). All patients who suffered from chronic HCV infection, as documented by a positive test screen for HCV RNA in serum, were included in this study. Signed informed consent was obtained from all of the patients before their inclusion. Liver biopsy and biochemical markers were performed the same day. Liver biopsy was performed at each center and analyzed by the resident pathologist. For all patients, ultrasound examination was performed before liver biopsy.

Information relating to the patients' demographics, risk factors, virological status, clinical examinations, clinical data [age at exposure to the virus, alcohol consumption and body mass index (BMI)] and biological data (virus genotype) was prospectively recorded at each center on the day of biopsy.

Patients were excluded if they suffered from another proven liver disease, such as autoimmune hepatitis or alcoholic liver disease, or if they had positive serology for hepatitis B or HIV. Patients for whom the date of infection was unknown were excluded as well.

The stored DNA samples from the 168 recruited patients were analyzed for the three mutations that are responsible for hypercoagulable states, that is, FV Leiden, PT20210 and MTHFR.

This study was approved by the local Helsinki ethics committees.

Definitions of slow and fast fibrosis

Fibrosis rates were calculated by dividing the fibrosis stage by the number of years of infection. We employed Poynard's fibrosis progression model in order to define our patients' fibrosis rate status and classified them as "fast fibrosers" or "slow fibrosers"[12]. According to the model,

Table 1 Patient characteristics by fibrosis rates (by Poynard)

	Slow fibrosers (n = 116)	Fast fibrosers (n = 52)	P value
Female (%)	37%	31%	0.43
Age (yr)	49.8 ± 10.5	43.9 ± 7.1	< 0.001
Age at infection (yr)	25.1 ± 10.8	25.2 ± 7.7	0.97
Infection duration (yr)	24.7 ± 8	18.7 ± 8.1	< 0.001
Alcohol intake ⩾ 50 g/d	7.1%	10.4%	0.53
BMI (kg/m^2)	23.8 ± 2.9	23.4 ± 3.1	0.5
Genotype (%)			0.49
1	74.3%	62.8%	
2	2.8%	2.3%	
3	16.5%	23.3%	
4	6.4%	11.6%	
Inflammatory score, grade (units)	1.3 ± 0.68	1.81 ± 0.69	< 0.001
Fibrosis stage (%)			< 0.001
0	14.7%	0%	
1	45.7%	9.6%	
2	29.3%	5.8%	
3	8.6%	48.1%	
4	1.7%	36.5%	

BMI: Body mass index.

the means of the fibrosis progression rates, as predicted by age and infection duration, are as follows: (1) patients who were infected at an age of less than 20 years were expected to develop cirrhosis after 40 years of infection; (2) patients who were infected in their third or fourth decade progress to cirrhosis after 35 years of infection; (3) patients in the fifth decade of life were expected to develop cirrhosis after 20 years of infection; and (4) patients who were older than 50 years were expected to become cirrhotic after 15 years of infection.

Histopathology

Liver biopsy examinations were performed at each center and analyzed by the local pathologist, wherein the fibrosis stage and activity grading were evaluated according to the METAVIR scoring system. Fibrosis was staged on a scale of 0-4: F0, no fibrosis; F1, portal fibrosis without septa; F2, few septa; F3, numerous septa without cirrhosis; and F4, cirrhosis.

The grading of activity that was assessed by the METAVIR system (based on the intensity of necroinflammatory activity, largely on necrosis) was scored as follows: A0, no histological activity; A1, mild activity; A2, moderate activity and A3, severe activity.

In order to assess liver biopsy quality, Regev quality criteria were used (fragment length of 15 mm or more, five or more portal tracts and one fragment). A biopsy that is between 10 and 15 mm in length and has less than five portal tracts or is fragmented is considered to be a fair quality biopsy, whereas a biopsy is considered to be of poor quality when it is less than 10 mm in length.

DNA collection and gene analysis

DNA samples were isolated from the peripheral blood of all patients. DNA extraction was performed using the QIAamp DNA blood kits and silica-membrane-based DNA purification (Qiagen, Germany).

Table 2 Frequency of hypercoagulation mutations (%)

Mutation type	Wild type	Heterozygote	Homozygote
Factor V Leiden (n = 159)	96.2	3.8	0
Prothrombin 20210 (n = 156)	92.3	7.1	0.6
MTHFR (n = 163)	35.6	50.9	13.5

Percentages of patients carrying each of the mutations by genotype. MTHFR: Methylene tetrahydrofolate reductase.

Analyses of gene mutations of FV Leiden, prothrombin G20210A and MTHFR C677T were performed *via* real-time polymerase chain reaction, using fluorescent hybridization probes (Dyn Diagnostics Roche, Israel) in a LightCycler instrument (Roche Diagnostics, Basel, Switzerland), specified as follows: FV Leiden: anchor: '5-LC-Red640-TGT CCT TGA AGT AAC CTT TCA GAA ATT CTG-PH, sensor WT: '5-GGC GAG GAA TAC AGG TAT-FL; PTG20210A: anchor: '5-LC-Red640-TGC TCC CAG TGC TAT TCA TGG AC-PH, sensor WT: '5-GAC TCT CAG CGA GCC TCA-FL; and MTHFR: anchor: '5-LC-Red640-CGC AGC TTT TCT TTG AGG CTG ACA-PH, sensor WT: '5-CGG GAG CCG ATT TCA TCAQ-FL.

Statistical analysis

All statistical analyses were performed using SPSS, version 17 (SPSS Inc., Chicago, Illinois, United States). The rate of fibrosis was calculated as the ratio of the fibrosis score to the duration of infection at the time of biopsy. This value was used for a univariate analysis of variance (ANOVA) in order to calculate whether the factors were significantly associated with the rate of fibrosis, and in a linear regression model to calculate the influence of each variable on the fibrosis rate. Fibrosis rates were subdivided into "slow fibrosers" or "fast fibrosers" for the construction of a multivariate logistic regression model that served to calculate the odds ratio (OR) for fast fibrosis.

Demographic differences between the "fast" and "slow" fibrosers were assessed using independent sample t test and ANOVA, whereas χ^2 test or Fisher's exact test were employed when appropriate, as designated in the tables.

RESULTS

One hundred and sixty-eight consecutive patients with available liver biopsies were recruited in this study. The average fibrosis rate in the entire cohort was 0.11 ± 0.17 fibrosis units per year. Demographic and disease-related data categorized by fibrosis rate are presented in Table 1. Patients who were categorized as "fast fibrosers" were significantly younger, had shorter disease duration, consumed more alcohol, and had a higher disease grade and stage according to histological analyses. HCV genotypes did not statistically differ between the two groups.

The frequencies of the three mutations that were analyzed in this cohort are specified in Table 2.

Table 3 Percentage of hypercoagulation gene mutation carriage by rate of fibrosis (by Poynard)

Gene	Mutation carriage	Percent of slow fibrosers	Percent of fast fibrosers	*P* value
PT20210	Wild type	94.5	86.9	0.18[1]
	Heterozygote	5.5	10.9	
	Homozygote	0	2.2	
Factor V Leiden	Wild type	95.5	98	0.67
	Heterozygote	4.5	2	
	Homozygote	0	0	
MTHFR	Wild type	35.6	36	0.80
	Heterozygote	52	48	
	Homozygote	12.4	16	

[1]Heterozygote and homozygote categories were unified for Fisher's exact test. PT20210: Prothrombin 20210; MTHFR: Methylene tetrahydrofolate reductase.

Table 4 Prothrombin 20210 mutation rates in various "fibrosis rate" models

Rate of fibrosis	PT20210 mutation in slow fibrosers (%)	PT20210 mutation in fast fibrosers (%)	*P* value[3]
Rate of fibrosis by Poynard	5.50	13	0.18
0.13 units/yr[1]	5.10	15.80	0.07
0.133 units/yr[2]	5	16.70	0.03

Various cut-offs of rate of fibrosis were employed on our cohort to differentiate between fast and slow fibrosers. [1]Rate of fibrosis according to Fishman *et al*[22]; [2]Rate of fibrosis according to Wright *et al*[10]; [3]*P* value calculated by Fisher's exact test. PT20210: Prothrombin 20210.

PT20210 carriers

Six patients (5.5% of 110 patients) of the "slow fibrosers" group were carriers of the PT20210 mutation, whereas, in the "fast fibrosers" group, 5 patients (10.9% of 46 patients) and one patient were heterozygous and homozygous, respectively, for the mutation (Table 3).

The occurrence of the PT20210 mutation among the "slow fibrosers" and "fast fibrosers" was not significantly different when the fibrosis rate was calculated according to the Poynard model; however, when we tested other cut-offs that have been used in the literature to differentiate slow and fast fibrosers, the difference became statistically significant (Table 4). Nevertheless, we used the Poynard model to define the slow and fast fibrosis groups in all of our calculations.

In a univariate analysis using the "rate of fibrosis" as the dependent variable and the PT20210 mutation status as the independent variable, while controlling for age, gender, BMI, amount of alcohol consumption, age of infection, and inflammation grade, we found that PT20210 status had a statistically significant association with the fibrosis rate ($P = 0.002$).

In order to explore the influence of PT20210 status, age, gender, age of infection, amount of alcohol consumption, BMI, and inflammation grade, we constructed a linear regression model with the above variables as the predictive variables, and the rate of fibrosis as the dependent variable (Table 5). These variables accounted for 44.8% of the variance in the fibrosis rate ($R^2 = 0.448$). The PT20210 status accounted for 9% of the fibrosis rate ($R^2 = 0.093$, $P = 0.002$).

Table 5 Linear regression analysis of rate of fibrosis

Predictive variable	R^2	*P* value
PT20210 status	0.093	0.002
Age	0.022	0.033
Gender	0.016	0.042
Age of infection	0.167	< 0.001
BMI	Not contributing	
Alcohol consumption	Not contributing	
Inflammation grade	0.121	< 0.001

PT20210: Prothrombin 20210; BMI: Body mass index.

Table 6 Multivariate analysis of the association between rate of liver fibrosis (slow fibrosers *vs* fast fibrosers) predicted by prothrombin 20210 mutation and various known parameters

Parameter	OR (95% CI)	*P*-wald[1]
Age	0.91 (0.86-0.96)	< 0.001
PT20210	4.76 (1.13-19.99)	0.033
Inflammation grade[2]	7.42 (3.07-17.95)	< 0.001

Variables removed from the model: gender, BMI and alcohol consumption. [1]The Wald test was used to assess the significance of each logit; [2]Inflammation was categorized: grades 0 and 1 compared with grades 2 and 3. PT20210: Prothrombin 20210; OR: Odds ratio; CI: Confidence interval.

In order to calculate the adjusted OR of the variables that affected liver fibrosis, we constructed a multivariate logistic regression model in a stepwise method. The variants that were included were PT20210 status, age, gender, BMI, alcohol consumption, and inflammation grade. The age of infection was not included, as it is used to define which patients are "fast fibrosers" in Poynard's model. We found that the presence of the PT20210 mutation corresponded with an OR of 4.76 ($P = 0.033$; 95% CI: 1.13-19.99) for "fast" liver fibrosis (Table 6).

Recent studies[13] have suggested that genotype 3 might be associated with the rate of fibrosis in hepatitis C patients. In order to account for this, we constructed an additional multivariate logistic regression model. This model included all universal variables that are known to affect the HCV fibrosis rate as well as genotype 3 status and the presence of the PT20210 mutation. Genotype 3 status was not a statistically significant predictor ($P = 0.25$), as opposed to PT20210, which remained in the model with an OR of 4.02 ($P = 0.048$; 95% CI: 1.01-16.00) (data not shown).

FV Leiden and MTHFR carriers

No significant association was found between FV Leiden carriage and fibrosis rate. Five patients (4.5% out of 110 patients with available gene analyses) of the "slow fibrosers" group were heterozygous for this mutation, as was only one patient (2% of 49 patients) of the "fast

fibrosers" group (Table 3).

Similarly, MTHFR was not associated with fibrosis rate in HCV patients (Table 3). Of the "slow fibrosers," 59 patients were heterozygous (52% of 113 patients with available gene analyses), whereas 14 (12.4%) patients were homozygous to the mutation. Among the "fast fibrosers", 24 (48% of 50 patients) patients were heterozygous for the mutation and 8 (16%) patients were homozygous for the mutation.

DISCUSSION

We have shown that carriage of the PT20210 mutation is related to an increased incidence rate of fibrosis in hepatitis C patients, whereas MTHFR or Factor V Leiden mutations are non-contributory.

The mean fibrosis rate in the "fast fibrosers" group was 0.23 ± 0.27 fibrosis units/year, which translates to 17 years of disease duration from infection to stage 4 cirrhosis. This rate was substantially faster than that of the "slow fibrosers" group (0.057 ± 0.037 fibrosis units/year), although classic contributing factors, such as BMI or alcohol consumption, did not substantially differ between the two groups.

In theory, enhanced coagulation may be an important factor in liver fibrosis. This hypothesis is supported by animal model studies, and, accordingly, a probable association between FV Leiden carriage and enhanced liver fibrosis in HCV patients was proposed[4,10]. The association of liver fibrosis with MTHFR carriage has not been directly examined, and the carriage of PT20210 has exhibited only an insignificant trend towards increased fibrosis in a single prior study[10].

In order to confirm that our results do not represent a type 1 statistical error, we employed three different cutoffs that have been previously used in the literature to differentiate fast from slow rates of liver fibrosis (Table 4) and eventually used the one that was the most stringent (the Poynard rate of fibrosis) in our calculations. Moreover, when the rate of fibrosis was used as a continuous variable (in the linear regression and univariate analysis models), the carriage of the PT20210 mutation was associated with faster fibrosis and explained up to 9% of the observed fibrosis rates when known factors that may affect fibrosis rate (age, age of infection, gender, alcohol consumption, BMI, and inflammation grade, which is more controversial) were controlled for.

Thus, although our cohort was relatively small, our findings suggest an impact of PT20210 mutation carriage on liver fibrosis in HCV patients. Interestingly, multiple mutations in more than one of the examined genes did not increase the fibrosis rate. We have not found a correlation between fibrosis rate and FV Leiden or MTHFR mutations. The relatively small number of patients who carried the FV Leiden mutation in our cohort may account for the lack of agreement between our findings and previous publications. With regard to MTHFR, one has to take into account folic acid and homocysteine levels, which were not examined in our cohort and may result in a non-hypercoagulation phenotype despite a procoagulation genotype. An additional limitation of our study may be the fact that none of the recruited patients had a clinical history of a hypercoagulable state, such as deep vein thrombosis. This may reflect a non-intentional selection bias and may have resulted in an underestimation of the hypercoagulation mutations in our cohort. Nevertheless, the percentage of patients who carried the PT20210 mutation in our cohort was at the high end of the range that has been reported in European cohorts, where the prevalence of the PT20210 mutation in the control groups is usually between 1%-3%, but may be as high as 6.5%[14]. PT20210 prevalence increases as one shifts from northern to southern Europe, and it is more prevalent in Caucasians as opposed to other ethnicities[15]. These two factors apply to our cohort and may at least partially explain the prevalence of PT20210 mutation carriage. Among the "slow fibrosers" group, 5.5% of patients carried the mutation, and this figure is within the reported prevalence of the PT20210 mutation in southern Europe.

The combined frequency of PT20210 among the entire cohort was above 7%; however, this statistic includes the "fast fibrosers" group, which we found to have a high frequency of PT20210 mutation carriage (13%). This signifies the importance of our findings. The PT20210 mutation was much more common in the "fast fibrosers" group, and, thus, the results of this study strengthen the notion that hypercoagulation causes faster fibrosis in HCV patients. In addition, the high percentage of PT20210 carriage in our cohort may partially explain why our results reached statistical significance, whereas Wright *et al*[10] demonstrated only a trend for a contribution of the PT20210 mutation to the fibrosis rate in HCV patients. In their cohort, although the number of patients was larger (n = 287), the prevalence of PT20210 mutation was only 4.5%.

Consistent with previous studies, we found that the age of infection and gender are related to fibrosis rate, whereas the HCV genotype had no effect on the rate of fibrosis, wherein the latter of these two has been controversial in the literature[2,13]. In contrast, inflammatory grade, which is considered to be a controversial factor regarding fibrosis rate, was determined to be a significant contributor to the fibrosis rate in our study. Because patients who suffered from alcoholic liver disease were excluded, only a small number of patients (< 8%) consumed large amounts of alcohol. This may account for the fact that we did not observe the well-known effect of alcohol consumption on liver fibrosis.

Although HCV patients who carry hypercoagulable gene mutations may account for only 5%-10% of all patients, millions of patients belong to this group around the world. Moreover, apart from the hypercoagulable states that relate to PT2010 and FV Leiden and were discussed here, other disease states may result in hypercoagulablity and perhaps in increased rates of liver fibro-

sis. Among these is the anti-phospholipid syndrome that has long been in debate with respect to its frequency and its effects in HCV patients. Specifically, anti-cardiolipin antibodies have been found in a sizable portion of HCV patients and are considered to be one of several autoimmune phenomena that are associated with this disease[16,17]. The implications of this disease on coagulation in this context are still under debate[18-21].

Ultimately, an analysis of gene mutations for hypercoagulability and the detection of hypercoagulable states in these patients may assist the clinician in the risk stratification of HCV patients according to fibrosis risk. Patients who are at high risk of liver fibrosis may be recommended to receive earlier anti-HCV therapy. With regard to the risk stratification of HCV patients according to the prevalence of the PT20210 mutation, a more specific and accurate method to stratify these patients may be found by assessing their prothrombin plasma levels, which may correlate better with the fibrosis rate.

This study lends additional support to the hypothesis that coagulation processes are involved in the pathogenesis of fibrosis in the liver. These accumulating reports should encourage the design of a larger prospective cohort study that will examine the impact of the various hypercoagulation states on liver fibrosis in HCV patients in particular, and in other fibrosis states of the liver in general. A better understanding of the pathophysiology of liver fibrosis may facilitate the development of novel therapeutic interventions that will help slow the rate of fibrosis.

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COMMENTS

Background

Cirrhosis is a major cause of morbidity and mortality in patients suffering from chronic hepatitis C virus (HCV) infection. Although various factors are known to affect liver fibrosis, it is still impossible to predict who will suffer from an increased rate of liver fibrosis among these patients. Recently, it has been suggested that HCV patients who possess hypercoagulation mutations [such as factor V Leiden (FV Leiden) mutation] may have an increased liver fibrosis rate.

Research frontiers

Hypercoagulable states have been hypothesized to play a role in organ fibrosis. In various inflammatory states, such as those of the lung or kidney, thrombosis and fibrin formation result in organ injury. It has been recently proposed that hypercoagulable states may be an additional contributing factor to liver fibrosis through several mechanisms, such as thrombotic events in small venous blood vessels in the liver, and hepatic stellate cell stimulation by thrombin. Increased fibrin deposition has also been demonstrated in animal models of liver fibrosis. These observations have been strengthened by a mouse model of liver fibrosis that demonstrated that the extent of fibrosis was much higher in mice carrying the FV Leiden mutation.

Innovations and breakthroughs

The study is the first to find a significant impact of an additional common hypercoagulation mutation, namely prothrombin G20210A (PTG20210A), on the rate of liver fibrosis in HCV patients.

Applications

The work strengthens the notion that hypercoagulation states affect the rate of liver fibrosis. If proven true in larger, prospective trials, HCV patients should be assessed for hypercoagulation mutations and should be regarded as a susceptible group for early cirrhosis, and receive early treatment against HCV. Additionally, better understanding of the role of hypercoagulation states in fibrosis may aid in the development of new therapies for organ fibrosis.

Terminology

Cirrhosis is a state of liver fibrosis with major morbidity and mortality in patients who suffer from liver diseases, in general, and particularly from hepatitis C. Hypercoagulation states such as PTG20210A, FV Leiden and methylene tetrahydrofolate reductase, are pathological conditions that cause an enhanced activity of the coagulation system and may expose patients to thrombotic events.

Peer review

The authors reported the association of fibrosis progression in chronic hepatitis C patients with PTG20210A mutation. This paper is interesting and informative.

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