Platelet-Derived Growth Factor Gene Polymorphism in Recurrent Hepatitis C Infection after Liver Transplantation

Ziv Ben-Ari,[1] Anat R. Tambur,[2] Orit Pappo,[3] Jaqueline Sulkes,[4] Vera Pravica,[5] Ian Hutchinson,[5] Tirza Klein,[6] Ran Tur-Kaspa,[1] and Eytan Mor[7]

Background. Recurrent hepatitis C virus (HCV) infection is particularly aggressive in the post liver transplantation setting, with rapid progression of liver fibrosis. Platelet-derived growth factor (PDGF) is reportedly involved in the pathogenesis of liver fibrosis. The aim of this study was to evaluate the possible contribution of molecular variants of the PDGF-B gene to recurrent HCV infection after liver transplantation.

Methods. DNA was extracted from peripheral blood mononuclear cells of 40 patients who underwent liver transplantation for chronic HCV infection and genotyped for polymorphisms in PDGF-B at positions +1135 (A to C) and +286 (A to G). Intrahepatic PDGF-B expression was detected by immunohistochemistry and assessed semiquantitatively. Forty-seven healthy individuals served as controls.

Results. Recurrent HCV infection occurred in 34 patients (85%) after a median interval of 10.5 months (range 1.5–60.0). A statistically significant difference was observed in the distribution of the PDGF-B gene polymorphism at position +1135, but not +286 between patients and controls ($P=0.05$). The A/A genotype occurred at a highly significantly increased rate in patients with recurrent HCV infection than in those without (64.7% vs. 16.67%, $P=0.0001$), and in patients with severe than in those with nonsevere recurrence (100% vs. 53.85%, $P=0.05$). The expression level of intrahepatic PDGF-B was found to be highly correlated with the fibrosis stage ($P<0.0001$). Further analysis yielded a highly statistically significant relationship between the PDGF-B gene polymorphism at position +1135 and clinical parameters of disease severity.

Conclusions. PDGF-B gene polymorphism appears to be associated with severe recurrent HCV infection after liver transplantation. PDGF-B may play an essential role in the development and progression of hepatic fibrosis. These findings, if confirmed, may have important therapeutic implications.

Keywords: PDGF, Gene, Polymorphism, Recurrent HCV, Liver transplantation.

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Hepatitis C virus (HCV)-related liver failure is the leading indication for orthotopic liver transplantation (OLT) worldwide. After liver transplantation, recurrence of HCV infection occurs in virtually all patients. The majority show HCV-induced allograft hepatitis after follow-up of at least 5 years (*1*). The natural history of the disease is characterized by progression to cirrhosis in 6% to 23% of patients at a median of 3 to 4 years after transplantation; the estimated cumulative probability of developing HCV-related graft cirrhosis at 5 years is 30% (*1–4*). The development of cirrhosis is associated with reduced graft and patient survival (*2*), and once cirrhosis is established, the 1-year actuarial risk for decompensation is 42% (*5*).

HCV-related disease progression is particularly aggressive in the posttransplantation setting. Fibrosis progresses significantly faster than in immunocompetent patients (*6*), suggesting that the time to the development of cirrhosis is shorter—approximately 9 to 12 years (*6*). The rate of progression is affected by a range of virus-, host-, and environment-related variables (*7*), namely: donor and recipient age, sex, and histocompatibility; year of transplantation; pretransplantation HCV RNA levels; viral genotype and quasispecies; use of immunosuppressive agents; and histological findings on the first liver biopsy.

Platelet-derived growth factor (PDGF), a cationic glycoprotein of 24 kDa, is reportedly involved in the pathogenesis of liver fibrosis (*8–11*). PDGF is released by endothelial cells, macrophages, fibroblasts, and vascular smooth muscle cells as a homo- or heterodimer composed of PDGF-A or PDGF-B chains. It exists as an AA, BB, or AB isoform, covalently linked by disulfide bonds (*12*). It has been implicated in the fibroproliferative response in various chronic inflammatory disorders (*13*). The human PDGF-B gene has been localized to chromosome 22 (q12.3–q13.1) and spans 12 kb of V-cis-related sequences interrupted by four intervening sequences (*14*). Novel single nucleotide polymorphisms were noted in the promoters, 5′-UTRs and introns of PDGF-B. Substitutions observed were G→A at position +286 (5′-UTR, accession number AF169594) and A→C at position

[1] Liver Institute and Department of Medicine D, Rabin Medical Center, Beilinson Campus, Petah Tiqva and Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel.
[2] Department of Histocompatibility and Immunogenetics, Rush Medical Center, Chicago, IL.
[3] Department of Histopathology, Rabin Medical Center, Beilinson Campus, Petah Tiqva and Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel.
[4] Epidemiology Unit, Rabin Medical Center, Beilinson Campus, Petah Tiqva and Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel.
[5] Immunology Research Group, University of Manchester, Manchester, United Kingdom.
[6] Tissue Typing Laboratory, Rabin Medical Center, Beilinson Campus, Petah Tiqva and Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel.
[7] Department of Organ Transplantation, Rabin Medical Center, Beilinson Campus, Petah Tiqva and Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel.
Address correspondence to: Ziv Ben Ari, M.D., The Liver Institute and Department of Medicine D Rabin Medical Center Beilinson Campus Petah Tiqva, 49100, Israel.
E-mail: gbenari@bezeqint.net.
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+1135 (first intron, accession number AF169595 (newly discovered by Ian Hutchinson and Vera Pravica, co-authors). There is no linkage disequilibrium between the two polymorphisms. In the liver, PDGF is released by inflammatory cells and promotes the proliferation of hepatic stellate cells (HSCs), the main source of extracellular matrix proteins (*15*). During hepatic fibrogenesis, PDGF is overexpressed (*16*), and HSCs undergo a process of activation, developing a myofibroblast-like phenotype (*17*) associated with increased proliferation and collagen synthesis (*18*). PDGF-B is the best characterized chemotactic factor for HSCs (*19–21*), and is recognized as their most potent mitogen.

The aim of the present study was to evaluate the possible association of molecular variants in the gene encoding PDGF-B with the risk of developing recurrent HCV infection after liver transplantation and with its severity.

PATIENTS AND METHODS

Patients

The initial sample included all 46 patients who underwent OLT for chronic HCV infection at Rabin Medical Center between 1992 and 2002. Six were later excluded because of concomitant alcoholic liver disease (n=2), a history of hepatitis B (n=2), or missing data (n=2). All participants had more than 1 year of follow-up (mean, 49.3±28.4 months). The diagnosis of recurrent HCV infection was based on the presence of viremia by quantitative polymerase chain reaction (PCR) assay, increased serum transaminase levels, and histologic findings at diagnosis of lobular hepatitis in association with hepatocyte necrosis and midzonal macrovesicular steatosis. The immunosuppressive regimen included cyclosporine, azathioprine, or mycophenolate mofetil (CellCept), in addition to corticosteroids in 17 patients and tacrolimus and corticosteroids in 23 patients. Episodes of acute, histologically proven cellular rejection were treated with three consecutive boluses of intravenous Solu-Medrol, 1.0 g/d, and steroid-resistant episodes were treated with OKT3.

Patient files were reviewed for demographic characteristics, pretransplant HCV RNA load and genotype, immunosuppressive regimen, rejection episodes, interval to recurrence, and clinical progression at follow-up based on levels of serum bilirubin, and alanine aminotransferase (ALT), presence of hepatic decompensation, retransplantation, and death due to recurrent HCV infection.

Severe recurrence was defined as stage 3–4 fibrosis, or the presence of recurrent fibrosing cholestasis or graft failure (*22*).

The control group included 47 healthy (HCV Ab-negative, HCV RNA-negative) volunteers (25 male, 22 female; mean age, 50.3±9.8 years) matched for sex and age (HCV Ab negative, HCV RNA negative) to the study group.

Genetic Polymorphism Assessment

The genetic profile of PDGF-B was analyzed in all patients.

DNA Extraction

Genomic DNA was isolated by proteinase K digestion of fresh peripheral blood mononuclear cells, followed by phenol extraction and ethanol precipitation. DNA samples were quantified and subjected to specific PCR reactions as described.

PDGF-B Gene Polymorphism

DNA was amplified by amplification refractory mutation system-polymerase chain reaction (ARMS-based PCR) (*23*) in a 10:1 reaction containing 200 M deoxyribonucleoside (dNTPs); 1.5 mM magnesium chloride; 8.5% sucrose (w/v); 0.25 units Taq polymerase; 5 M specific control primers and 1 M internal control primers. PDGF-B +286 primers were designed (by Pravica V & Hutchinson I) to detect an A to G polymorphism: 5′-AAGGCCGGAACAGCTGAAA-3′ and GGTCCGTCTGCCCGCCC/T; PCR product size was 323 bp. PDGF-B +1135 primers were designed to detect an A to C polymorphism: 5′-TGTTCTCGGGTTCCCAAAGG-3′ and ATTCATTACCTTCGCCCCCC/A; PCR product size was 263 bp. The internal control primers amplified the human growth hormone gene: sense primer 5′-GCCTTCCCAACCATTCCCTTA-3′ and antisense primer 5′-TCACGGATTTCTGTTGTGTTTC-3′; PCR product size was 429 bp. PCR included 1 cycle of 95°C×1 min; 10 cycles of 95°C×15 seconds, 69°C×50 seconds, 72°C×40 seconds; and 20 cycles of 95°C×20 seconds, 59°C×50 seconds, 72°C×50 seconds.

Method validation was performed using alternative techniques: restriction fragment length polymorphism.

Histological Assessment

One pathologist (O.P.) blindly reviewed all hepatic specimens of the patients for overall necroinflammatory activity (grade 0 to 12) and fibrosis (stage 0 to 4) according to Knodell's score. Intercurrent disease processes, such as acute cellular rejection, cytomegalovirus infection, biliary obstruction, and ischemia were ruled out by serologic, immunohistochemical, radiological and endoscopic studies.

PDGF-B Immunohistochemistry Staining and Scoring

Immunohistology was performed on deparaffinized liver biopsy sections.

Briefly, sections (4 μl) of formalin-fixed, paraffin-embedded tissues were immunostained with mouse antihuman PDGF-B (μU 376-UC, BioGenex, San Ramon, CA) diluted 1:100. Antigen retrieval was performed before application of the primary antibody. The tissue sections were placed in a bath with citrate buffer pH=6.0 and microwaved in a pressure cooker on high power (900–1000 W) for 13 min until the pressure cooker was fully pressurized. Thereafter, the microwave level was reduced to 40% for another 5 min. Endogenous peroxidase reactions were blocked using Dako's blocking kit (Dako Co., Carpinterie, CA). Sections were then incubated with the primary antibody for 45 min followed with the Dako LSAB + kit peroxidase, which consists of labeled streptavidin biotin reagents. Reactive sites were revealed by incubation with DAB (3.3-diaminobenzidine) (Dako). Sections were counterstained with hematoxylin. Positive controls were sections of squamous carcinoma and negative controls were the liver biopsies run concurrently without the primary antibody.

The intrahepatic PDGF-B expression was evaluated semiquantitatively according to the number of cells that stained positively (*24*).

Virological Assays

HCV RNA was tested by nested reverse-transcription polymerase chain reaction assay (RT-PCR) (Cobas Amplicor HCV Monitor Test, Roche Diagnostic Systems, Branchburg, NJ). Analytical sensitivity of the assay was 600 IU/ml. HCV genotypes were determined by a line-probe hybridization assay (INNO-LiPa Innogenetics, Ghent, Belgium) directed to the 5′ untranslated regions of the different HCV genotypes.

Statistical Analysis

Pearson and Spearman correlation coefficients and the significance for it (p) were calculated between the variables.

Chi-squared test or Fisher exact test were used, as appropriate, to analyze statistically significant relationships between categorical variables (i.e., study vs. control group, recurrence, mutations +286 and +1135), and chi-squared test for equal proportions was used to analyze the distribution of each type of mutation between the study and control groups. Student *t* test was used to analyze statistically significant differences in mean continuous parameters between the groups. To analyze statistically significant differences in mean continuous parameters between more than two groups of categorical variables, analysis of variance was used with Duncan multiple comparison option. Due to the small sample size in some subgroups, a non-parametric Kruskal-Wallis test was also done. Multivariate stepwise logistic regression models were fitted to the data in order to predict mortality, disease recurrence or disease severity. A p value less than or equal to 0.05 was considered statistically significant.

RESULTS

The baseline characteristics of the patients in this study are presented in Table 1: mean age was 52.7±9.6 years; 52.5% were male. HCV genotypes were available for 30 patients: 26 (86.6%) were genotype 1, 3 were genotype 2a and one was genotype 3a. Mean pretransplant HCV RNA load was 414,954.8±880,991.4 IU/ml. The immunosuppressive regimen was based on cyclosporine in 35% of the patients and on tacrolimus in 65%. The rejection rate was 45% (in 18 patients). Of these, 12 patients developed mild rejection, five moderate, and only one patient severe (treated with OKT3).

Recurrent HCV infection developed in 34 patients (85%) after a median interval of 10.5 months (range 1.5–60.0 months); in 8 patients, the recurrent infection appeared early (<12 months) and was severe. Mean serum ALT level at recurrence was 139.7±121.0 U/L, and mean serum bilirubin, 1.8±2.0 mg/dl; mean necroinflammatory score was 5.3±2.4, and mean fibrosis score, 1.7±1.1. Six of the 34 patients (17.6%) showed histologically proven cirrhosis during the follow-up period. The mean duration of follow-up was 49.8±28.4 months. The remaining six patients did not have histological recurrence of HCV. In all of them, liver biopsy was done during follow-up because of an increase in serum ALT level, which was found to be due to other causes and ultimately resolved.

TABLE 1. Baseline Characteristics of Patients (n=40) (mean±SD)

Age (years) (mean)	52.7±9.6
Sex (F/M)	47.5%/52.5%
Immunosuppression (cyclo/tacrolimus)	35%/65%
Rejection episodes (n)	18 (45%)*
Recurrent HCV (n)	34 (85%)
Alanine aminotransferase (U/L)**	139.7±121.0
Bilirubin (mg/dl)** (mean)	1.8±2.0
Necroinflammatory score**	5.3±2.4
Fibrosis score**	1.7±1.1
Cirrhosis (n)	6 (17.6%)
Viral genotype 1 (n)	26 (86.6%)***
Viral load (IU/ml)	414,954.8±880,991.4
Time to recurrence (months) (median)	10.5 (range, 1.5–60.0)
Severe recurrence (n)	8 (23.5%)
Follow-up (months) (mean)	49.8±28.4

* Of these, 12 patients developed mild rejection, 5 moderate, and 1 severe.
** At diagnosis of recurrent HCV after transplantation.
*** HCV genotypes were available for 30 patients.

Phenotypic Expression

The genotype and allele frequencies were determined in patients and controls (Table 2).

A statistically significant difference was observed in the distribution of the PDGF-B gene polymorphism at position +1135 between the patients who underwent liver transplantation for HCV and the control group ($P=0.05$). However, no such statistically significant difference was observed at position +286.

Table 3 summarizes the phenotypic expression deduced from the gene polymorphism in PDGF-B in the patients with and without HCV infection after liver transplantation and in the patients with severe and non-severe recurrent disease. The allelic polymorphism of PDGF-B at position +1135 examined the presence of an A or C nucleo-

TABLE 2. Frequency of PDGF-B polymorphism

Polymorphism	*n*	Genotype	Frequency (%)	*P* value
PDGF-B +286	40	AA	20	NS
		AG	64	
HCV		GG	16	
			52	
			48	
Control	46	AA	24	
		AG	59	
		GG	17	
			53	
			47	
PDGF-B +1135	40	AA	59	0.05
		AC	34	
HCV		CC	7	
			76	
			24	
Control	43	AA	36	
		AC	51	
		CC	13	
			58	
			42	

TABLE 3. PDGF-B gene polymorphism in patients after liver transplantation for HCV infection (n = 40)

Posttransplantation HCV recurrence	PDGF-B gene polymorphism					
	+1135 (%)			+286 (%)		
	AA	AC	CC	AA	AG	GG
No recurrence (n=6)	1 (16.67)	1 (16.67)	4 (66.66)	3 (50.0)	3 (50.0)	0
Recurrence (n=34)	22 (64.71)	11 (32.35)	1 (2.94)	5 (14.7)	23 (67.65)	6 (17.65)
P value		0.0001			NS	
Severe recurrence (n=8)	8 (100)	0	0	1 (12.5)	6 (75.0)	1 (12.5)
Nonsevere recurrence (n=26)	14 (53.85)	11 (42.31)	1 (3.84)	4 (15.38)	17 (65.38)	5 (19.23)
P value		0.05			NS	

tide in this position, which translated directly into three phenotypic expressions. A highly statistically significant difference was observed between the patients who developed recurrent HCV infection after liver transplantation and those who did not ($P=0.0001$). The majority of patients (64.71%) with recurrent HCV infection exhibited the A/A genotype, 32.35% exhibited the A/C genotype, and 2.94% the C/C genotype; by contrast, 16.67% of the patients who did not develop HCV recurrence exhibited the A/A genotype, 16.67% the A/C genotype, and 66.66% the C/C genotype (Fig. 1).

A highly statistically significant difference was also observed among the patients with recurrence, by severity of disease ($P=0.05$). All patients with severe recurrence had the A/A genotype. Of the patients with non-severe recurrence, 53.85% exhibited the A/A genotype, 42.31% the A/C genotype, and 3.84% the C/C genotype (Fig. 1).

The allelic polymorphism of PDGF-B at position +286 examined the presence of an A or G nucleotide in this position, which translated directly into three phenotypic expressions. No statistically significant difference was observed between the patients who developed a recurrent HCV infection and those who did not (Table 3). Within the group of patients with a recurrence, there was no statistical difference between those with severe and non-severe recurrent disease (Table 3).

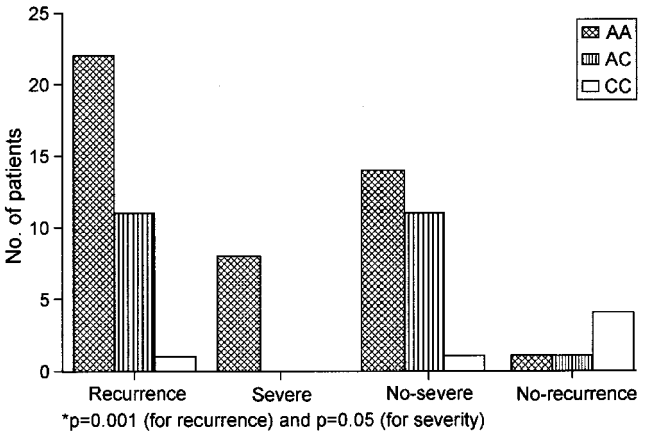

FIGURE 1. PDGF-B gene polymorphism (+1135) and recurrent HCV infection.

Intrahepatic PDGF-B Immunohistochemistry

There was a statistically significant difference between the PDGF-B gene polymorphism at position +1135 and the intrahepatic immunohistochemistry for PDGF-B ($P=0.027$). PDGF-B immunoreactivity was found in mesenchymal cells of portal areas and fibrous septa localized diffusely in the cytoplasm compartment. A highly statistically significant correlation was found between the intrahepatic immunohistochemistry for PDGF-B and the fibrosis score ($P<0.0001$). The number of positive cells increased with progression of fibrosis (Figs. 2 and 3) (Table 4). No statistically significant correlation was found between PDGF-B immunohistochemistry and the necroinflammatory score. A statistically significant correlation was found between PDGF-B immunohistochemistry and the serum bilirubin level ($P=0.003$), serum ALT ($P=0.0002$), severity of recurrence ($P=0.0008$), death due to HCV ($P=0.0004$).

Relationship between the PDGF-B Gene Polymorphism and Clinical Parameters

On univariate analysis, highly statistically significant correlation was noted between the PDGF-B gene polymor-

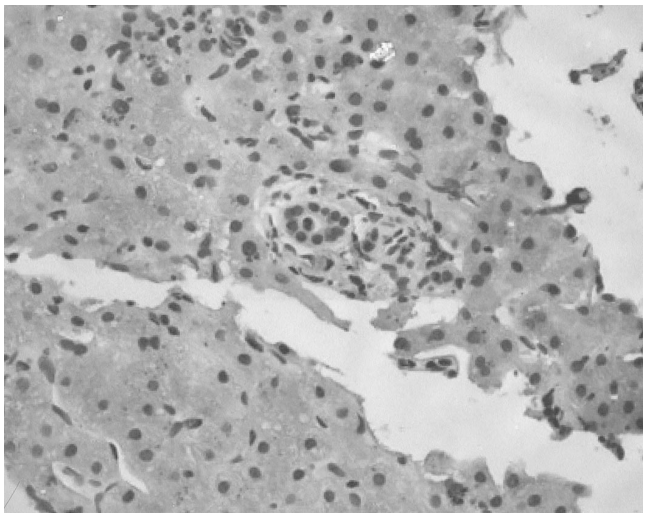

FIGURE 2. Immunohistochemical detection of PDGF-B in liver biopsy in a patient with recurrent HCV infection and a fibrosis stage 0. No PDGF-B immunoreactivity was found.

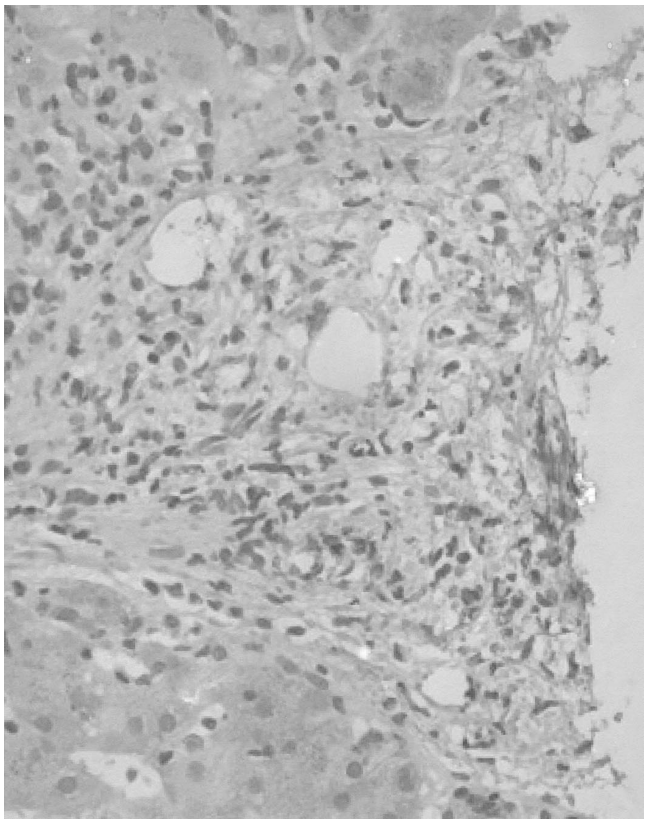

FIGURE 3. Immunohistochemical detection of PDGF-B in liver biopsy in a patient with recurrent HCV infection and a fibrosis stage 3. The number of positive cells increased with the progression of fibrosis.

TABLE 4. PDGF-B expression levels in liver samples with different stages of fibrosis[a]

Stages of fibrosis	*n*	Expression levels (mean±SD)
F_0	1[b]	3.6±1.1
F_1	11	5.3±3.2
F_2	14	9.2±3.4
F_3	2	13.4±5.1
F_4	6	19.6±5.9

[a] $P < 0.0001$.
[b] n = number of cases in each fibrosis stage of patients with recurrent HCV infection.

phism at position +1135 and recurrent HCV infection ($P=0.001$), serum ALT level ($P=0.007$), and serum bilirubin level ($P=0.04$) at recurrence, necroinflammatory score ($P=0.03$), fibrosis score ($P=0.001$), cirrhosis ($P=0.003$), severe recurrence ($P=0.022$), and death due to recurrent HCV infection (8 patients) ($P=0.06$) (Table 5). Factors found to have no association were immunosuppressive regimen, rejection episodes, viral load, and viral genotype before transplantation, time to recurrence and retransplantation. Although multivariate analysis was performed, no statistical predictive factors were entered into the model due to sample size restrictions (small number of deaths or recurrence events).

No statistically significant correlation was noted between the PDGF-B gene polymorphism at position +286 and the studied clinical parameters (Table 5), except for the necroinflammatory score ($P=0.04$).

TABLE 5. Relationship between PDGF-B gene polymorphism and clinical parameters

Parameter	*P* value +1135	*P* value +286
Sex	NS	NS
Age	NS	NS
Immunosuppression (cyclosporine/tacrolimus)	NS	NS
Rejection episodes	NS	NS
Recurrence	0.001	NS
Alanine aminotransferase	0.007	NS
Bilirubin	0.04	NS
Necroinflammatory score	0.03	0.04
Fibrosis score	0.001	NS
Immunohistochemistry	0.027	NS
Cirrhosis	0.003	NS
Viral genotype	NS	NS
Viral load	NS	NS
Time to recurrence	NS	NS
Severity	0.022	NS
Death due to hepatitis C virus	0.06	NS

NS, not statistically significant.

DISCUSSION

Variables associated with the rapid disease progression of HCV reinfection after transplantation are under intensive investigation (*7*). To the best of our knowledge, this is the first study to examine the role of molecular variants of the gene encoding PDGF-B in this process.

We focused on two PDGF-B gene polymorphisms, at positions +1135 (A to C) and +286 (A to G). A statistically significant difference was noted in the distribution of the PDGF-B polymorphism at position +1135 between the study and control groups ($P=0.05$). No such relationship was found for position +286. Further analysis yielded significant relationships of the polymorphism at position +1135 with both risk of recurrence and its severity. Specifically, the A/A genotype occurred at a significantly higher rate in patients with recurrence than in those without (64.7% vs. 16.67%, $P=0.0001$), and in patients with severe recurrence than in those with non-severe recurrence (100% vs. 53.85%, $P=0.05$). In addition, the polymorphism at position +1135 was associated with higher clinical parameters of disease at recurrence (serum ALT, bilirubin, fibrosis score, cirrhosis, and severity of infection). Due to the small number of events (death or recurrence), the model could not be fitted into a multivariate context. By contrast, the PDGF gene polymorphism at position +286 did not correlate with recurrent HCV infection (p=NS).

These findings might help to shed light on the mechanisms underlying the accelerated course of HCV infection in liver transplant recipients compared to immune-competent individuals (*6*) both before and after the development of cirrhosis (*5*). The posttransplantation period is known to be characterized by a faster rate of fibrogenesis until graft cirrhosis develops and a greater risk of subsequent decompensation

than before transplantation (*3*, *6*, *25–27*). Nevertheless although most patients develop recurrent HCV within 5 years, some maintain minimal to moderate liver damage whereas others advance rapidly to end-stage disease and graft failure (*1–3*, *6*, *28*). One of the key mediators of progressive liver disease are HSCs (*15,18*), they are considered to be the main source of extracellular matrix protein in the liver (*19–21*). The present study was prompted by evidence indicating that the PDGF-B is one of the most potent mitogenic factor for HSCs (*19–21*). PDGF is overexpressed during active hepatic fibrogenesis and may be involved in the transformation of HSCs to myofibroblast-like cells in vivo (*16*). These findings were supported by the study of Pinzani et al. (*8*), who reported markedly increased PDGF-A and -B chain mRNA expression in cirrhotic livers, indicating the functional involvement of PDGF/PDGF-R (receptor) in liver fibrogenesis. Malizia et al. (*9*) also noted a high expression of PDGF-A and -B in mononuclear and proliferating ductal cells in livers with diseases of various etiologies. Indeed, we have found a statistically significant relationship between PDGF-B gene polymorphism at position +1135 and the intrahepatic immunohistochemistry for PDGF-B, and a highly statistically significant correlation between the immunohistochemistry for PDGF-B and the fibrosis stage. Therefore, PDGF-B may play an essential role in the development and progression of hepatic fibrosis in recurrent HCV infection after liver transplantation. The management of PDGF activity by antagonists or by soluble PDGF-B receptor that inhibit PDGF signaling and PDGF-induced proliferation in culture of HSC (*29*) might prevent aggressive liver fibrosis and improve prognosis in patients with recurrent HCV infection after liver transplantation.

Our data emphasize the potential importance of PDGF-B gene polymorphism in recurrent severe HCV infection and the role of PDGF-B in the development and progression of hepatic fibrosis. Further analysis in a larger cohort of patients is needed to confirm our results. These findings, if confirmed, may ultimately help clinicians therapy in selected patients at risk to design preemptive preventive

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