ORIGINAL PAPER

RJME
Romanian Journal of Morphology & Embryology
http://www.rjme.ro/

Immunohistochemical study of stellate cells in patients with chronic viral hepatitis C genotype 1

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Abstract

Introduction: Hepatic stellate cells (HSC) are key-players in the pathogenesis of liver fibrosis, inducing collagen deposition and abnormal extracellular matrix remodeling. *Aim*: The purpose of this study was to identify the stellate cells using immunohistochemical techniques and to establish if there is a correlation between the expression of stellate cells and the clinical and histological parameters in patients with chronic viral hepatitis C. *Materials and Methods*: The studied group included 30 patients with chronic viral hepatitis C genotype 1, in whom a liver biopsy was performed previous to the antiviral treatment. After the histological analyze, the biopsy was stained with an anti-SMA antibody (Dako, Carpinteria, CA). The amount of positive stained area was determined using an arbitrary semiquantitative score from 1 to 4. *Results*: Our observations suggest that there is a strong correlation between the stellate cells activity, evaluated using a semiquantitative score, and the stage of liver fibrosis (rs=0.76, p<0.001). Also, our study revealed a direct correlation, but less intense, with the necro-inflammatory activity (rs=0.39, p=0.03), the steatosis degree (rs=0.428, p=0.01) and the value of alanine aminotransferase (rs=0.4, p=0.03). The age and the viremia level were not correlated with the activity of the stellate cells. *Conclusions*: This study suggests that the transition of stellate cells from inactivated state to the state of highly fibrogenic cell is influenced mainly by the histological liver modifications (necroinflammatory activity and steatosis) and less by clinical parameters (age, sex) or the viremia level.

Keywords: chronic viral C hepatitis, stellate cells, immunohistochemistry.

Introduction

The hepatic virus C infection represents one of the major health problems of the contemporary world, with around 180 million infected persons at global level, each year adding 3–4 million new cases [1, 2]. In the European area, Romania is rated with a high prevalence, being estimated at around 3.5% [3, 4]. The prognosis of patients with chronic hepatitis C infection is directly linked with the hepatic fibrosis evolution towards cirrhosis and the development of its complications.

The hepatic stellate cells (HSCs) activation represents the trademark of liver fibrosis. In the healthy liver, stellate hepatic cells are the inactivated non-parenchymatous cells, whose main functions are to store vitamin A and probably to maintain a normal basal membrane. The activation of stellate cells plays a key role in liver damage, and refers to the transition from inactivated cells rich in vitamin A to the level of highly fibrogenic cell. Stellate cell proliferation occurs in areas with high liver damage, being preceded by an influx of inflammatory cells and followed by the accumulation of extra-cellular matrix. Activation consists of two major phases: the initiation (the pre-inflammatory stage) and perpetuation (inflammatory stage).

Hepatic stellate cells (HSCs) express almost all of the major components necessary for pathological degrading of the matrix, and therefore they play a key role in matrix production, as well as in its degradation. An increasingly large family of matrix metalloproteases has been identified, enzymes depending on calcium levels, which are degrading in a specific way the collagens and the non-collagen sublayers. The hepatic C virus replication releases factors that modulate stellate cell expression, increase the synthesis of procollagen I and III, also decreasing the fibrinolytic activity of the matrix proteinase [5].

Our aim was to identify the stellate cells using immunohistochemical techniques and to establish if there is a correlation between the expression of stellate cells and the clinical and histological parameters in patients with chronic viral hepatitis C.

Materials and Methods

Patient characteristics

We prospectively included a number of 30 random patients with chronic viral hepatitis C, admitted in the I[st] Medical Clinic – Gastroenterology, University of Medicine and Pharmacy of Craiova, for clinical

evaluation prior to antiviral therapy with peginterferon and ribavirin. The patients included in this study have respected the inclusion criteria for antiviral treatment:

1. Virusological criteria: detectable levels of ARN VHC;
2. Biochemical criteria: ALT levels normal or increased;
3. Morphological criteria: chronic hepatitis – Metavir score A≥2 and F≥1; Knodell–Ishack ANI≥6 and F1≥ 1;
4. Age ≤65 years.

All patients gave informed consent in order to participate in the study. All necessary approvals were obtained from the hospital ethic commission.

In all patients included in the study, quantitative ARN VHC has been determined using Cobas Amplicor VHC Monitor method (Roche Diagnostics), as well as VHC genotyping using Linear Array VHC Genotyping test (Roche Diagnostics). All 30 patients included in our study had viral genotype 1 present.

The control group consisted of seven-tissue samples prelevated from embryonic liver samples, with an age between 10 and 38 weeks.

Histopathologic study

The histopathologic and immunohistochemical study was performed on hepatic tissue samples prelevated by transcutaneous liver biopsy. The liver biopsy was performed with an automatic gun (Bard Autovac) with tru-cut needle with diameter of 1.4 mm. The coagulation tests and the number of thrombocytes were within normal levels for all the biopsied patients.

The obtained liver biopsy fragments were formalin fixated and paraffin embedded. Slices of 4–5 μm were cut from the paraffin blocks and were stained using Hematoxylin–Eosin (HE), Masson's trichrome, Goldner–Szekely green light trichrome, as well as the Gömöri technique. The histopathologic assessment was performed by an experienced pathologist (M.V.C.). The activity and liver fibrosis were evaluated using Metavir and Knodell scores. The stage of fibrosis was graded on a scale from 0 to 4, according to the Metavir score: without fibrosis – stage 0; mild/moderate fibrosis at portal space level – stage 1; marked portal or periportal fibrosis with occasional septa but with preserved architecture – stage 2; numerous septa with portal-portal or portal-central bridging fibrosis and architectural distor-tion, but without cirrhosis – stage 3; and stage 4 – cirrhosis [6]. Macrovesicular steatosis was graded 0–3 based on the percent of hepatocytes in the biopsy involved (0 is none; 1 is up to 33%; 2 is 34 to 66%; 3 is >66%) [7].

Immunohistochemical study

The same paraffin blocks used for the histological technique, were also used for the immunohistochemical examination, being displayed on a glass slide, previously treated with poly-L-lysine. To determine the amount of activated HSCs, the biopsy was stained with an anti-SMA antibody (Dako, Carpinteria, CA). As a primary antibody, we used the HHF35 clone, antihuman mouse, diluted 1:200, and as the secondary antibody we used IgG horse anti-mouse, diluted 1:500. The immuno-staining, obtained using the DAB chromogen and Hematoxylin counterstained, was emphasized at cytoplasm and membrane level. The amount of positive stained area was determined using an arbitrary semi-quantitative score from 1 to 4: 1 – blood vessel staining; 2 – portal or periportal staining; 3 – mild portal-portal or portal–central bridging staining; and 4 – intense portal-portal and portal-central staining.

The histopathologic and immunohistochemical aspects were emphasized using the Nikon Eclipse E200 microscope with 4× magnification and attached optical correction devices with magnification 10×, 20×, 40×. The most significant images were taken using a digital video camera Nikon DS–Fi1 and stored directly on the computer using the Lucia Net software, version 1.16.5.

Statistical analysis

Results of quantitative variables are expressed as mean ± standard error of the mean. Correlation between necroinflammatory activity, fibrotic stages, degrees of steatosis, age, viral titers and semiquantitative analysis of α-SMA positive stellate cells was evaluated using the Spearman's correlation test. Comparisons between groups were performed using the Mann–Whitney test. A p-value <0.05 was considered statistically significant. Statistical analyses were performed with the Statistical Package for the Social Sciences (SPSS) version 17.0.

Results

Characteristics of the patients with chronic viral hepatitis C enlisted in our study are illustrated in Table 1.

Table 1 – ***Patients' characteristics (mean ± standard deviation)***

Sex (M/F)	14/16
Age [years]	48.03±9.85
Body mass index [kg/m^2]	26.93±3.87
Aspartate amino transferase [U/L] N<35 U/L	97.85±160.79
Alanine amino transferase [U/L] N<39 U/L	117.32±145.23
HCV viral load [×10^5 U/L]	4.46±2.16
HCV genotype 1	30
Fibrosis stage (Metavir score)	
Mild to moderate (F1–F2)	17
Severe (F3–F4)	13
Necroinflammatory activity (Metavir score)	
A1/A2/A3	11/15/4
Steatosis grade (Brunt score) 0/1/2/3	5/13/6/6

All the patients have shown viral genotype 1. Repartition of the group based on the degree of liver fibrosis (mild *vs.* severe) was balanced.

Histopathology

Microscopic examination of the sections evidentiated the patterns of necrosis, inflammation and fibrosis. Identifying hepatocytary lesions and the presence of inflammatory infiltrate was regarded as activity,

Distribution of inflammatory cells varied from case to case; however, in all instances it was characterized by the presence of a dense monocytary infiltrate in the interlobular spaces. Any number between none and all

interlobular spaces were affected. The dimensions of interlobular spaces were normal or enlarged because of the flux of inflammatory cells. We observed the expansion of the portal fibrous stroma, which pushed the adjacent structures without harming them (Figure 1).

Inflammatory infiltrate was composed of lymphocytes and plasmocytes. We also identified macrophages containing intracellular necrotic detritus, at interlobular spaces level.

We regarded the presence of apoptotic hepatocytes and the inflammatory infiltrate at the contact region between the parenchyma and the mesenchyme stroma as interface hepatitis (piecemeal necrosis) and was quantified as follows:

- Light (focal, few port spaces);
- Medium (focal, most port spaces);
- Moderate (continuum, less than 50% of tracts and septae) (Figure 2);
- Severe (continuum, more than 50% of tracts and septae).

Fibrosis quantity identified within the lesions varied from absent, up to lesions compatible with a diagnosis of hepatic cirrhosis (Figure 3).

We characterized fibrosis by the presence of expansions of the portal spaces with or without the formation of septae. Fibrotic septae unified the central veins either with adjacent port spaces (P–C), or with portal veins (P–P). At this level, we identified a mononuclear inflammatory infiltrate (Figure 4).

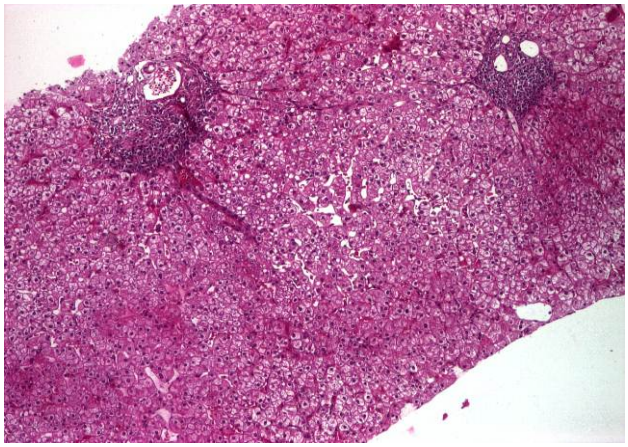

Figure 1 – ***Chronic hepatitis with the presence of an inflammatory infiltrate limited to the interlobular space. Hematoxylin–Eosin staining, 40×.***

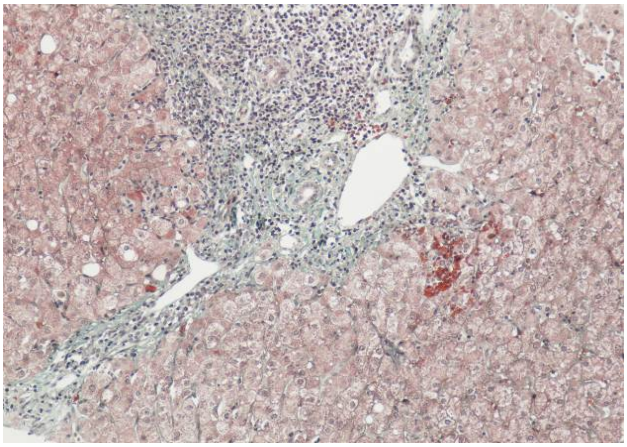

Figure 2 – ***Chronic hepatitis with the presence of moderate interface hepatitis. Goldner–Szekely staining, 100×.***

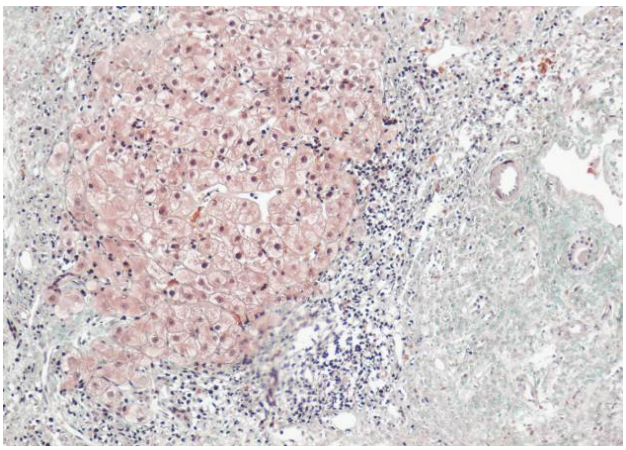

Figure 3 – ***Cirrhotic nodule with abundant inflammatory infiltrate. Goldner–Szekely stain, 100×.***

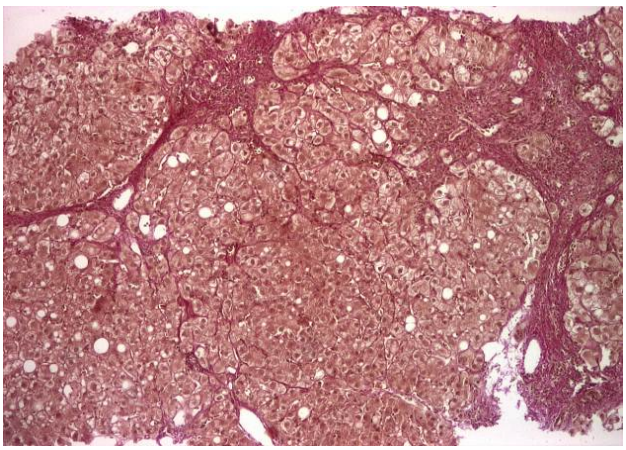

Figure 4 – ***Chronic hepatitis; P–P and P–C fibrosis. Van Gieson staining, 200×.***

Steatosis was identified by the presence of optic hollow vesicles within the cytoplasm of hepatocytes, due to lipid dissolving while paraffin inclusion, with clear limits, of varied sizes. Macrovesicular steatosis, appeared due to progressive lipid accumulation, caused the nucleus to be pushed to the periphery. It was evaluated in three grades: light (0–30% of all hepatocytes), moderate (30–60% of all hepatocytes) and severe (over 60% of all hepatocytes).

Immunohistochemistry

Fetal liver was rich in progenitor cells, which are positive to alpha actin; being present both in vascular walls, and within myofibroblast-like cells of the parenchyma.

We noticed an increase in the number of alpha actin positive cells in patients with moderate and severe hepatitis, as well as cirrhosis, as compared with minimal invasion cases.

With regard to the distribution of α-SMA-positive HSCs, in the control group (fetal liver) we found very few α-SMA-positive HSCs, only along the sinusoids, mostly in the peripheral zones of the hepatic lobule close to the portal spaces. Hepatocytes and cells of the

epithelial duct turned out to be negative to α-actin (Figure 5). In the HCV–C groups, α-SMA-positive HSCs were strongly and diffusely immunostained, being correlated with the degree of fibrosis (p<0.001) (Figure 6). Therefore, in the group of patients with mild and moderate fibrosis (F0–F2 groups), we have noticed positive staining for α-SMA within the perivenular, intermediate zone (Figure 7) and periportal area (Figure 8). In patients with severe fibrosis and cirrhosis, α-SMA expression varied from moderate to severe in the portal spaces and fibrous septa (Figures 9–11).

The activity of the stellate cells was strongly correlated with fibrosis (Spearman's coefficient =0.76, p<0.001) (Figure 12). We have also found a correlation with the value of alanine aminotransferase (Spearman's coefficient =0.4, p=0.03), steatosis (Spearman's coefficient =0.428, p=0.01) and also with necro-inflammatory activity (Spearman's coefficient =0.39, p=0.03) (Figures 13–15). We have not found a correlation between the expression of stellate cells and age (Spearman's coefficient =0.03, p=0.04) or viremia (Spearman's coefficient =0.05, p=0.03).

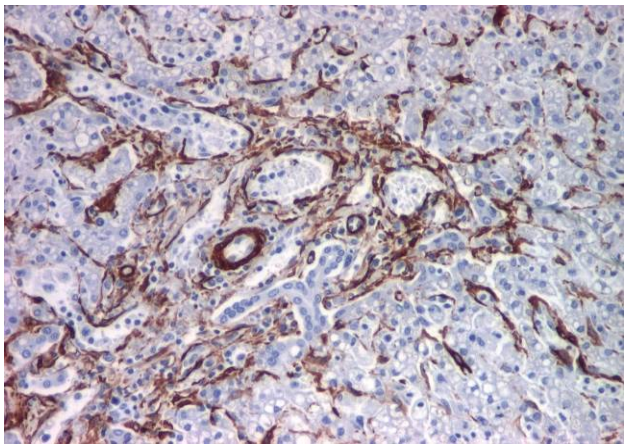

Figure 5 – ***Smooth muscle's immunostaining with alpha-actin within the fetal liver, 200×.***

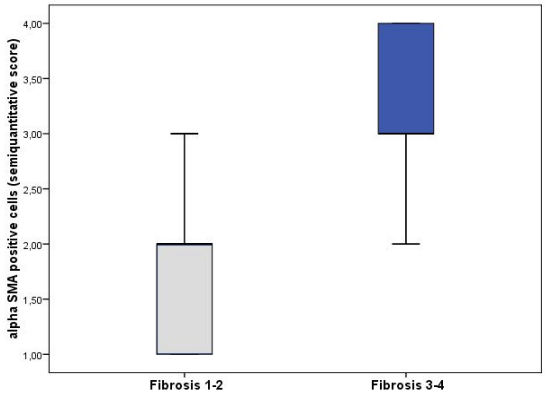

Figure 6 – ***Comparison of alpha-SMA immuno-reactivity score between patients with slight fibrosis (F1–F2) and severe fibrosis (F3, F4).***

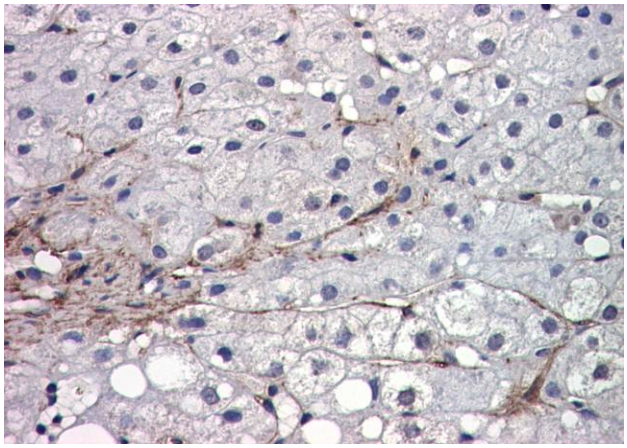

Figure 7 – ***Immunostaining with alpha-actin within the portal space (F1). 400×.***

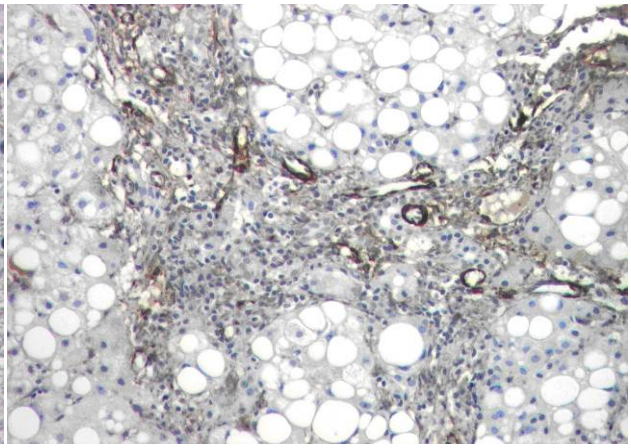

Figure 8 – ***Immunostaining with alpha-actin within the portal space and occasional septa (F2), 200×.***

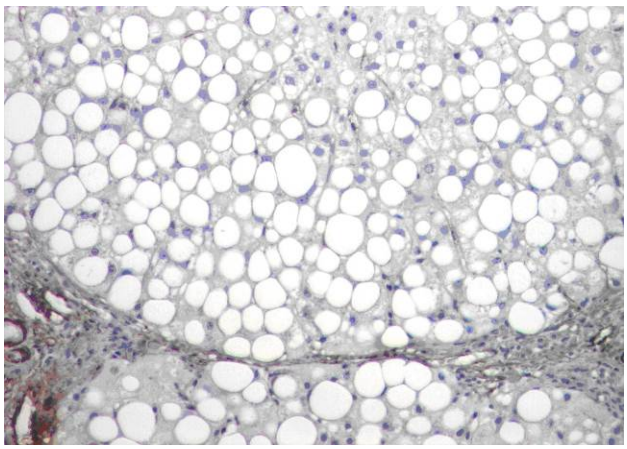

Figure 9 – ***Immunostaining with alpha-actin within porto-portal septa, 200×.***

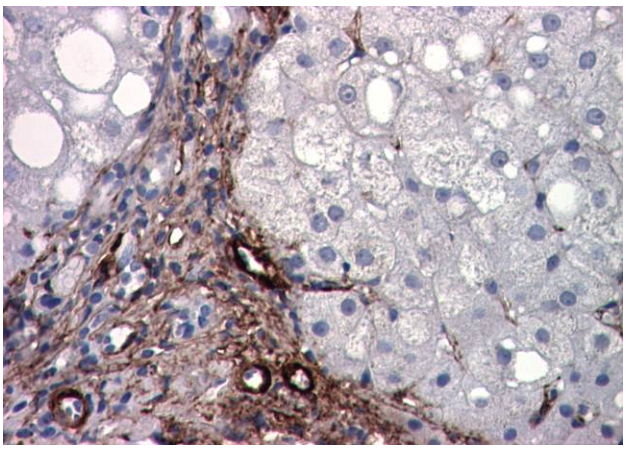

Figure 10 – ***Immunostaining with alpha-actin within the portal spaces and fibrous septa (F4), 400×.***

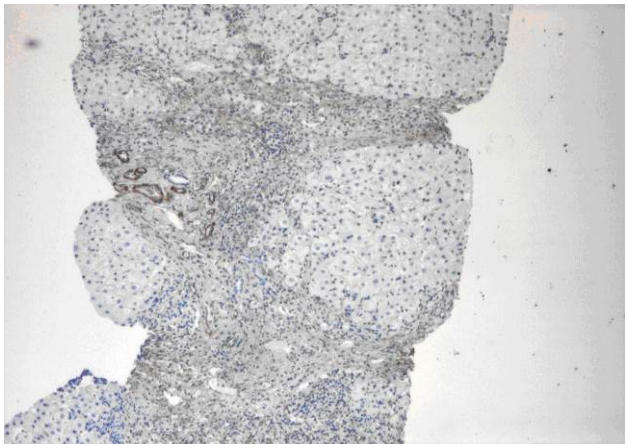

Figure 11 – ***Hepatic cirrhosis: immunostaining with alpha-actin (general view), ×40×.***

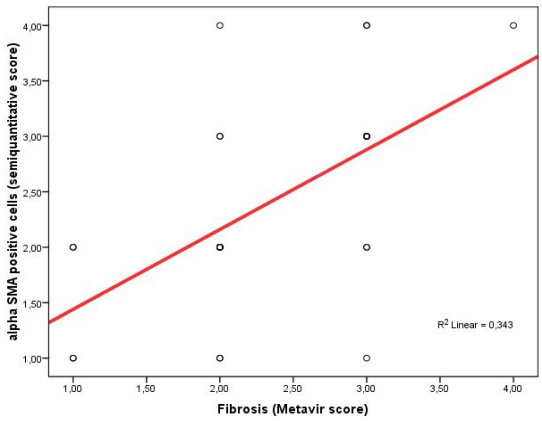

Figure 12 – ***Correlation of alpha-SMA immunoreactivity score with fibrotic stages in patients with chronic hepatitis C. Standardized coefficient =0.76, p<0.001.***

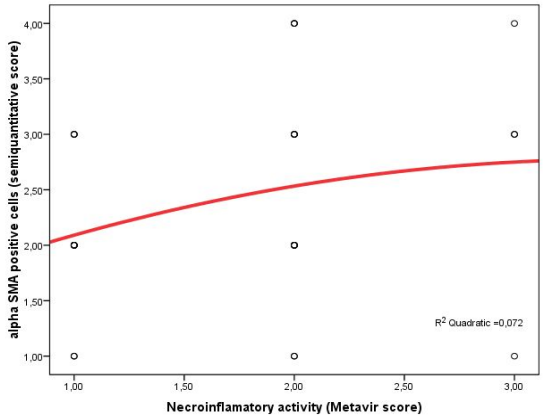

Figure 13 – ***Correlation of alpha-SMA immunoreactivity score with the ALT value in patients with chronic hepatitis C. Standardized coefficient =0.4, p=0.03.***

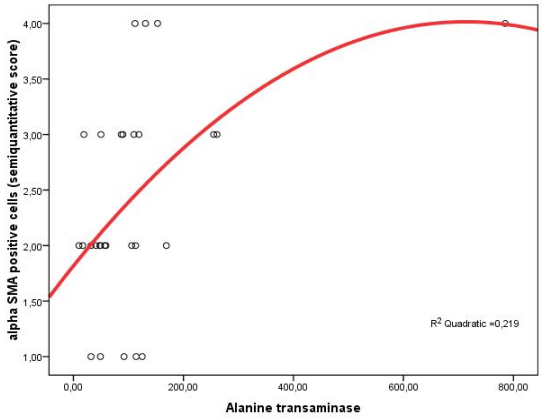

Figure 14 – ***Correlation of alpha-SMA immunoreactivity score with necroinflammatory activity in patients with chronic hepatitis C. Standardized coefficient =0.39, p=0.03.***

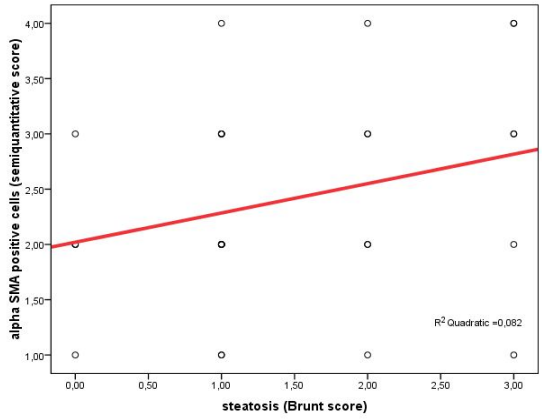

Figure 15 – ***Correlation of alpha-SMA immunoreactivity score with the degree of steatosis in patients with chronic hepatitis C. Standardized coefficient =0.428, p=0.01.***

Discussion

Stellate cells approximately account for 5–8% of total cells in normal liver. They are located in the perisinusoidal space of Disse, in between the fenestrated endothelium of sinusoids and the hepatocytes, with a higher frequency in the periportal area compared to the centrilobular area. The fact that the stellate cells play an important role in the development of liver fibrosis and its progression towards cirrhosis is well-documented [8–13]. In stress conditions and injuries (e.g. hepatitis C virus), the stellate cells are activated within myofibroblasts, which are highly fibrogenous cells.

HSCs can express α-smooth muscle actin (α-SMA) upon activation, and the expression of α-SMA by HSCs was considered a marker of their activation to myofibroblast-like cells [12, 14]. Alpha actin is an important marker of activated stellate cells and is able to precede the process of fibrosis. Alpha actin positivation in stellate cells in chronic hepatitis and cirrhosis reflect their activation towards myofibroblast like cells, as well as their role in extracellular matrix remodeling, and consecutively in fibrogenesis.

Nowadays, fibrosis is known as a dynamic and continuous process in which stellate cells play a key role. In their activation, a key role is played by chemokines, and they in turn are able to secrete chemokines, thus creating a "vicious circle" between fibrogenesis and the stimulation of inflammatory cells, hence resulting in an accentuation of hepatic lesions.

In the fetal liver, alpha-actin stains the fusiform cells at portal level. During 13th to 20th weeks of fetal life, rare stellate cells are observed within hepatic canalicules. The alpha-actin is staining the hepatic arteriolar walls, but not the sinusoids. Alpha-actin does not stain hepatocytes. Myofibroblasts are absent in healthy liver, but are common in liver with different degrees of fibrosis. Recent studies have shown that the patients with chronic viral hepatitis C have increased activation of HSCs, as shown by expression of α-SMA [14–17]. Most studies demonstrate a strong correlation between stellate cells activity and the degree of fibrosis [15–18], but the statistical data regarding the correlation between stellate cells activity and the degree of necro-inflammatory activity, steatosis, viremia or age remain controversial. Activation of stellate cells, identified by mesenchymal markers such as alpha-actin, indicates a correlation with the severity of fibrosis, while the interaction with the necroinflammatory activity remains low [17–21]. The data from medical literature shows a decrease of stellate cells activity in patients who received antiviral treatment with interferon [16, 19].

In our study, we have discovered a strong correlation between the stellate cell activity and the stage of fibrosis in patients with chronic viral hepatitis C. We have also observed a correlation between the activity of stellate cells and the degree of necroinflammatory activity. This data may suggest the roles played by the inflammation in the stellate cell activity, with a major part in initiating and progression on liver fibrosis. Concerning the hepatic steatosis, our studied group consisted mostly of mild steatosis. Our data proves to be concordant with those from literature regarding patients infected with viral genotype 1 [22]. The degree of hepatic steatosis was also correlated with the activity of stellate cells, which may explain the influence of hepatic steatosis in the progression of liver fibrosis in patients with chronic viral hepatitis C.

The presence of stellate cells in the portal space and within the septae has a predictive value in the development of fibrosis, especially after hepatic transplants [23, 24].

The identification of the stellate cells activity as a major source of extracellular matrix in liver fibrosis may be a start point for new antifibrinogenic therapy.

Conclusions

Alpha-SMA proves to be a very accurate marker for HSC differentiation to myofibroblast-like cells. The stellate cell activity is directly correlated with the stage of fibrosis, necroinflammatory activity and hepatic steatosis. Identifying the activated stellate cell opens new perspectives in early diagnosis of liver fibrosis, as well as in future antifibrinogenic therapies.

Acknowledgements

This paper was supported through the CNCSIS Research Grant No. 789 entitled "The evaluation of genetic and immunohistochemic markers of hepatic fibrogenesis as predictive factors of antiviral therapy response in chronic viral C hepatitis" and No. TD–151, entitled "Predictive factors of sustained virological response at hepatitis C patients".

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Received: December 5th, 2010

Accepted: February 25th, 2011