Experimental & Clinical Hepatology

Received: 2011.06.27
Accepted: 2011.07.15
Published: 2011.12.15

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

Prevalence of nonalcoholic fatty liver disease and its biochemical predictors in patients with type-2 diabetic mellitus

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Summary

Background: Nonalcoholic fatty liver disease (NAFLD) is emerging as the most common chronic liver condition in the Western world and it is commonly associated with type 2 diabetes mellitus (DM). The aim of this study to determine the prevalence of NAFLD, and identify the predisposing factors in Type 2 DM patients with NAFLD.

Material/Methods: A total of 258 patients of type 2 DM were included in the prospective study in a tertiary referral hospital. Patients with characteristic findings on ultrasonography were considered as having fatty Livers. They were divided into fatty liver (Group I) and non fatty liver group (Group II) and were further evaluated by measurement of body mass index, liver function tests and lipid profile.

Results: Out of 258 type 2 diabetic patients, 167 (64.7%) patients had fatty liver on ultrasonography. BMI, Waist-hip ratio and triglyceride levels in the group I was significantly higher than Gp II. An increase in the levels of ALT, AST, total cholesterol, LDL and a decrease in HDL was observed in Gp I as compared to Gp II.

Conclusions: The prevalence of NAFLD is common among in type-2 diabetic patients and it increases with the rising incidence of obesity. Obesity as well as elevated liver enzymes, triglyceride and cholesterol are significantly raised in NAFLD patients with Type 2 DM. It highlights the importance of routine liver function test and lipid profile in subjects with type 2 DM and should be more closely observed for NAFLD and liver complications.

Key words: **Type-2 diabetes mellitus • NAFLD • LFT • lipid profile**

Full-text PDF: http://www.expclinhep.com/fulltxt.php?ICID=969154

Word count: 1873
Tables: 1
Figures: –
References: 39

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Background

Nonalcoholic fatty liver disease (NAFLD) is a type of chronic liver disorder which is gaining significant importance worldwide. NAFLD represents a spectrum of conditions characterized histologically by macrovesicular hepatic steatosis and occurs in those who do not consume alcohol in amounts generally considered to be harmful to the liver [1]. Numerous studies show that it is hepatic component of metabolic syndrome [2,3] whose central features are peripheral insulin resistance, obesity, hyperinsulinemia, hypertriglyceridemia and hypertension [3,4]. NAFLD is strongly associated with obesity,type-2 diabetes mellitus(Type 2 DM) and hyperlipidema [3,5]. The aetiology of liver function test (LFT) derangements in type 2 diabetes mellitus may be quite varied. Non-alcoholic fatty liver disease (NAFLD) is often associated with LFT abnormalities. The reported prevalence of NAFLD in type 2 diabetes mellitus ranges from 30–75% and is almost universally associated with morbidly obese subjects with diabetes [6,7]. The prevalence of NAFLD is increasing in India and other Asian countries because of the westernization of the lifestyle, such as a high-fat and high-calorie diet and less physical activity [8]. The aim of this case control study was to determine the prevalence of NAFLD in Type 2 DM, and identify the predisposing factors in Type 2 DM patients with NAFLD and those with out NAFLD.

Material and Methods

The patients with diagnosed type 2 diabetes mellitus who attended medicine out patient departments for the master health checkup scheme, and those who were admitted in inpatient wards during the period between June 2010 to May 2011 were included. Diabetes mellitus was defined as per the WHO diagnostic criteria with fasting plasma glucose >126 mg/dl was used for patient selection. For each subject, demographic details, clinical findings and laboratory results were recorded, including age, gender and duration of diabetes. These patients were subjected to a detailed history and physical examination with emphasis on symptoms of liver disease, duration of diabetes, family history of diabetes mellitus and other cause of liver disease. Overweight was defined as a body mass index (BMI) between 23 and 25 kg/m^2, and obesity as Body Mass Index (BMI) equal or above 25 kg/m^2 [9]. Patients were considered centrally obese if the waist circumference was greater than 80 cm in females and 90 cm in males [10].

Exclusion criteria were 1) an alcohol intake of more than 40 g per week 2) usage of drugs known to cause steatosis including amiodarone, corticosteroids, tamoxifen, methotrexate 3) Patients with Pre existing liver disease (hepatitis B surface antigen or Anti HCV positive) 4) patients who gave previous history of liver disease, Fatty liver was diagnosed on the basis of diffuse hyper echoic echo texture, increased echo texture compared with kidneys, vascular blurring and deep attenuation by using abdominal ultrasonography

The following laboratory tests were recorded: liver function tests [Aspartate aminotransferase (AST), Alanine aminotransferase (ALT)]; hemoglobin A1c (HbA1c), fasting blood glucose (FBS), lipid profile (total cholesterol(TC), and high density lipoprotein (HDL), low density lipoprotein (LDL), triglyceride(TG)) and viral profile (HBsAg, Anti HCV antibody).Liver enzymes were defined as abnormal if the concentration exceeded the upper limit of normal for the reference range The BMI, waist/Hip ratio, HbA1c,AST, ALT, AST/ALT ratio,FBS, Lipid profiles were compared between Type 2 DM patients with fatty liver (Group I) and those patients without fatty liver (Group II). Institutional ethics committee permission was obtained and informed written consent was obtained from all the patients included in the study. Data was collected using a standardized proforma and analyzed using the SPSS 16.0 version statistical software. Data are presented as mean ± standard deviation (SD) or as a percentage (%). Statistical analysis was carried out for study parameters between the two groups (NAFLD and non-NAFLD) using student's T test. P value less than 0.05 was considered statistically significant.

Results

A total of 258 patients of type 2 diabetes mellitus were studied, of which 146 (56.9%) were those of male patients and 112 (43.4%) were those of female patients. The mean duration of the diagnosis of Type 2 DM was 10.7±7.2 years. Comparison of anthropometric, liver enzymes, lipid profile between fatty liver and non fatty liver groups is presented in Table 1. The frequency of NAFLD within different age groups was not significant (p<0.78). None of the patients had histories of alcohol consumption. 167 (64.7%) patients had fatty liver whereas 91 (35.6%) had no fatty liver on ultrasound. The average liver size was 17.2±3.1 cm in Gp I and 13±2.4 cm in Gp II. Serum AST, ALT and lipid profile, were also significantly higher (P value <0.001) in Gp I patients. Serum HDL level is significantly low in Gp I patients (P<0.001). The average BMI and waist hip ratio were high in Gp I than Gp II and the differences were found to be statistically significant (P value <0.001).

Discussion

NAFLD, which is characterised by a wide spectrum of liver pathology ranging from mere liver steatosis to the more severe non-alcoholic steatohepatitis, resembles alcohol-induced liver disease, but develops in subjects who are not alcohol consumers and have negative tests for viral and auto-immune liver diseases [11,12]. The prevalence of NAFLD is high in conditions associated with insulin resistance, such as obesity, type-II diabetes mellitus, dyslipidemia and the metabolic syndrome [5].

The prevalence of fatty liver varies from 10 to 20% in the general population and increases to 50–75% in subjects with type 2 DM [6]. We have estimated that approximately 59.5% of patients with type 2 DM had a fatty liver in our study. This result is similar to the findings from other studies [13–15]. In the present study, increased weight, BMI and waist measurement were more prevalent in patients with NAFLD, situations in which the insulin resistance is a predominant factor [16,17].

BMI was significantly higher in Gp I patients than Gp II (p=0.001). Obesity is the most common entity associated with NAFLD that has been reported in studies [18]. Higher levels of triglycerides were more observed in Gp I patients which may possibly reflect a greater accumulation of fatty acid into the liver, higher insulin resistance and a greater tendency to develop into NAFLD [19].

Dyslipidemia has been reported in 20 to 92% of patients with NAFLD Elevated serum triglycerides and low HDL

Table 1. Comparison between NAFLD and Non-NAFLD.

Details	Group I (NAFLD)	Group II (Non-NAFLD)	P-value
Age (years)	54.3±4.1	54.8±7	0.78
BMI (kg/m^2)	30.6±2.39	27.45±2.69	<0.001
Waist hip ratio	1.275±0.34	0.905±.0.845	<0.001
FBS (mg/dL)	172±53.1	170.6±48.4	0.734
Hb A1c (%)	7.34±0.62	7.4±0.21	0.642
ALT (IU/L)	36.16±20.35	19.61±5.91	<0.0001
AST (IU/L)	38.35±19	24.57±5.52	<0.001
AST/AST ratio	1.28±0.36	1.14±0.29	0.836
TC (mg/dL)	201±50.29	164.42±35.56	<0.001
TG (mg/dL)	199.62±63.36	153.12±19.5	<0.001
HDL (mg/dL)	38.29±6.32	44.46±7.64	<0.001
LDL (mg/dL)	119.12±39.8	89.64±28.84	<0.001

NAFLD – nonalcoholic fatty liver disease; BMI – Body Mass Index; FBS – fasting blood sugar; ALT – alanine aminotransferase; AST – aspartate aminotransferase; TC – total cholesterol; TG – triglyceride; HDL – high density lipoprotein; LDL – low density lipoprotein.

cholesterol is the features of the metabolic syndrome [14]. In our study also Gp I have showed that higher level of triglycerides and lower HDL compared to Gp II.

In fact, 30 to 100% of patients diagnosed with NAFLD have been shown to be obese. The prevalence of NAFLD in obese individuals is 76% as compared with 16% in non-obese individuals.[20] The greater the degree of obesity, the greater the prevalence and severity of NAFLD [18,21]. However, individuals with normal BMI may also be affected by NAFLD, particularly those with abdominal obesity [22].

In present study, the waist/hip ratio was significantly high for Gp I compare Gp II patients (P<0.001). This results support the ratio reflects abdominal fat distribution and it has been shown in a previous study that there is a significant correlation between waist/hip ratio and the degree of hepatic steatosis, even in patients with normal BMI [23]. In recent studies have demonstrated that a relatively small weight loss of 8 kg in patients with poorly controlled T2DM reversed their hepatic steatosis and normalized fasting plasma glucose concentrations, rates of hepatic glucose production, and hepatic insulin responsiveness [24].

Although initial studies emphasized that NASH occurred mostly in women, more recent study have shown that NAFLD occurs with equal frequency in men [25] as is also seen in this study. The FBS, HbA1C levels were similar between the patients with and without NAFLD and those with and without fibrosis. Absence of correlation between glycemic control and NAFLD may denote to an indirect relation between these two conditions. The correlation of liver fat content with insulin sensitivity independent of body fat content is found in the absence of clinical steatosis individuals, and most patients with NAFLD are hyperinsulinemic and more insulin resistance compared with non-steatotic healthy subjects. While the relevance of NAFLD and insulin resistance is invariable, their potential relationship has been reviewed [26].

Age has been found to be an independent risk factor for both NAFLD and fibrosis in some studies [27]. In our study, mean age of the patients and the duration of diabetes were not significantly different between Gp I and Gp II. The ultimate diagnosis of NAFLD is based on the histologic examination of liver biopsy. However, it is an invasive and costly procedure and is associated with several complications. Radiologic imaging of the liver with ultrasound computed tomography or magnetic resonance imaging has an adequate threshold for the detection of fatty liver. We have used ultrasound to identify NAFLD which has a sensitivity and specificity of 89% and 93%, respectively, in detecting liver steatosis. In fact, imaging tests are insensitive when the degree of steatosis is less than 33% [28,29].

The presence of mildly raised serum liver enzymes, including Alanine aminotransferase (ALT), Aspartate aminotransferase (AST) are the most frequent and sometimes the only laboratory abnormality found in NAFLD patients [30,31]. Even elevated ALT levels within normal range could predict incident diabetes mellitus, suggesting that hepatic fat accumulation is a contributing factor for conversion to diabetes mellitus in men at risk [32,33]. Recently, a number of prospective cohort studies showed that this marker predicted the development of metabolic complications independently [34,35]. An AST to ALT ratio >1 might predict more severe disease [36] with a greater probability of fibrosis. In present study, AST to ALT ratio was <1. However, it can be taken as the sign of fatty infiltration of the liver [37]. Since advanced fibrosis is unlikely to regress spontaneously; these patients have the risk of progression to cirrhosis, hepatocellular carcinoma and liver cell failure. It is therefore important to identify type 2 diabetes patients who are at the highest risk.

The relationship between fatty liver, impaired glucose tolerance, diabetes mellitus and hyperlipidemia is well established [38]. Our study showed increased triglyceride levels in diabetic fatty liver group as compared to non fatty liver group and the results were statistically significant (P value

<0.001). Elevated triglycerides were also found to be a predictor of fatty liver in diabetic population.

Insulin resistance does not seem to be correlated with the presence of NAFLD among T2DM patients. The diagnosis of NAFLD in our study was based on ultrasonography findings may therefore overlook subjects with small amount of fat infiltration of the liver. In fact, hypertriglyceridemia have been strongly correlated with liver fat accumulation [39].

Conclusions

We conclude that, NAFLD is commonly seen in Type-2 diabetic patients, and its prevalence is likely to increase with the rising incidence of obesity and diabetes. The independently associated risk factors for NAFLD are the raised BMI, waist/hip ratio, as well as higher levels of liver enzymes and elevated Serum triglyceride and serum cholesterol are significantly raised in NAFLD patients than in non fatty liver type 2 diabetic patients. The high prevalence of severe derangements also highlights the importance of performing routine liver function test and serum triglycerides and cholesterol monitoring in subjects with type 2 diabetes mellitus. Type 2 diabetic patients and NAFLD should be actively required to treat and should also be monitored for the development of NAFLD and its complications.

Conflict of interest

None

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Experimental & Clinical Hepatology

Received: 2011.10.26
Accepted: 2011.11.14
Published: 2011.12.15

Morphologic alterations found in esophageal microvascular endothelial cells in cirrhotic patients

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Source of support: University of Buenos Aires, Argentina

Summary

Background: Portal hypertension (PH) constitutes the most frequent complication associated with chronic liver disease, like cirrhosis. This complication is frequently accompanied by severe bleeding, originated from the rupture of esophagogastric varices. This PH, created by a hyperdynamic circulation, is transmitted to the azygos vein due to the impossibility of blood to pass through the damaged liver. It has been recently described that there is a dynamic control of the microcirculation attributed to substances from the endothelial cells function (ECs): vasoconstrictor and vasodilator substances, creating a balance in normal tissue blood flow.

Material/Methods: Nine patients (7 males and 2 females; mean age 50 years old) with alcoholic cirrhosis with non bleeding esophageal varices and (PH) (Group I) were studied and compared with nine control subjects (Group II). Cirrhosis was confirmed by percutaneous liver biopsy assessed by usual clinical and biochemical analysis. Upper fibroscopy was carried out with a video endoscope Fujinom EG 200HR. Esophageal biopsies were carefully obtained from the esophageal wall. 2 or 3 cm over the highest varix, fixed in glutaraldehyde and observed in electronic microscope Zeiss.

Results: Electronic microscopy of the biopsies from group I evidenced capillary dilatation with aneurysmatic aspect in microcirculation. In some zones of the esophageal submucosa, the endothelial cells were hyperplasic and some protruded into the lumen, occasionally occlude it. Vacuolization, were capillary vasodilatation and thrombosis were also detected. Some biopsies showed a lacunar aspect with some cells floating inside this structure, some of them with necrotic characteristics.

Conclusions: It is possible that prolonged PH causes anomalous local circulation, through the inverted blood flow direction in esophageal perforating veins. The finding of important morphological alterations in endothelial cells from esophageal veins can be the expression of this anomalous circulation.

Key words: esophagus • portal hypertension • varices

Full-text PDF: http://www.expclinhep.com/fulltxt.php?ICID=969158
Word count: 2127
Tables: –
Figures: 4
References: 26

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Background

Different kinds of complications are commonly associated with chronic liver disease. Portal hypertension (PH) constitutes the most frequent complication found in cirrhotic patients. This syndrome is frequently accompanied by severe bleeding, a life threatening event originated from ruptured gastroesophageal varices.

The pathophysiology of PH relies basically on the presence of a splanchnic hyperdynamic circulation. The increase in blood pressure is then transmitted through porto systemic collaterals to the azygos veins circulation, including the esophageal, venous system. Portal pressure is probably one of the principal factors for the development of structural alteration in the esophageal venous wall.

Using portal hypertensive rats, Sarfeh et al. [1], described, endothelial hypertrophy, budding of endothelial cells, capillary narrowing and tortuosities in their gastric microvasculature, not present in control rats. In the last years, the presence of a dynamic control of tissue microvasculatory perfusion, has been attributed to the existence, of vasoconstrictor and vasodilator substances produced by endothelial cells, endothelins – ETs- and nitric oxide (NO), which create a balance in normal blood flow for adequate cell nutrition [2].

Moller et al., [3] demonstrated increases in ET-1 plasma concentration in cirrhotic patients, during severe decompensation. Veglio et al. [4] did not confirm these data, but Uchihara et al. [5] presented conclusions similar to those of Moller et al.

These endothelial cell (EC) secretions are known to regulate the local blood tissue perfusion in a paracrine-autocrine manner [2]. Hence, alterations in the vascular endothelium could be responsible, for the development of tissue disturbances.

According to the present concepts of ECs functions, the aim of this investigation was to determine the morphological conditions of esophageal microvascular ECs subjected, to portal pressure in cirrhotic patients, suggesting the presence of possible functional disorders and adding some pathophysiological data on the formation and rupture of varices.

Material and Methods

Nine patients (7 males, 2 females; mean age 50 years old) with portal hypertension due to alcoholic liver cirrhosis with esophageal varices were included in this study (group I). The diagnosis of liver cirrhosis was established on the basis of clinical, ultrasonographic and biochemical examinations. Exclusion criteria were hepatocellular carcinoma, other esophageal diseases and decompensated cirrhosis (Child Pugh class C), prothrombin time less than 60%, and platelet count less than 60.000/mm^3.

Group II: nine patients (6 males, 3 females; mean age 58 years old) without esophageal, gastric, duodenal or liver pathology were included as a control group. These patients had indication for an esophageal endoscopy because they presented dyspepsia and pyrosis.

Video endoscopic studies were performed (Fujinom EG 200 HR or EG 200 CT, processor EPX201 or a Pentax EG 2940 with a EPM 330 P), and biopsy specimens from esophagus mucosa were obtained 2 or 3 cm over the highest varix observed. Biopsy samples were fixed in glutaraldehyde in buffer of Na cacodylate 0.1 M, included in epoxy resin (Poly/Bed) and with Vinyl acetate and Reynald salt, and observed under an electronic microscope (electron transmission microscopy Zeiss).

No complications were observed in the procedures and patients returned home after 24 hours observation in the hospital.

Serology screening for hepatitis, B and C, herpes simplex, cytomegalovirus, Epstein Bar virus and HIV were determined using standard methods. All the patients were negative for these determinations.

This study was performed according to the principles of the Declaration of Helsinki, and was approved by the Institutional Review Board and the Bioethical Committee of our Institution. Written consent was obtained from all the patients.

Results

Electronic microscopy of the biopsies from group I evidenced capillary dilatation with an aneurysmatic aspect in some zones in the esophageal sub mucosal in microcirculation.

The endothelial cells were hyperplasic and some protruded into the lumen (Figure 1), occasionally occluding it. Vacuolization (Figure 2), capillary vasodilatation and thrombosis were also detected. Some biopsies showed a lacunar aspect with some cells floating inside this structure, necrotic ECs (Figures 3, 4).

In the esophageal lower third, high resolution microscopy confirmed the presence of capillaries with aneurysmatic dilatation and, hyperplasic endothelial cells, some of which presented necrotic characteristics.

Hepatic biopsies from the group I patients showed the presence of disorganisation of parenchymal normal structure of acini. We found presence of important fibrosis in almost all cases and regeneration nodules showing traces of morphologically normal cells. The hepatic microcirculation was markedly altered by the presence of regenerative nodules and fibrotic bands. Intake of alcoholic beverages existed in our group I patients presented a 10 to 15 years history of drinking. Eight of our patients had moderate hepatomegaly (4 to 6 cm under lower right rib) and all showed by ultrasonography splenomegaly, that was palpable in only two of them. Ultrasonography also showed a small amount of intraperitoneal fluid.

Endoscopy of Group II control patients showed normal esophageal mucosa and three cases of slight gastric.

Discussion

This study aimed to observe the possible existence and magnitude of morphological endothelial cell alterations in the lower third of the esophagus, cirrhotic portal hypertensive

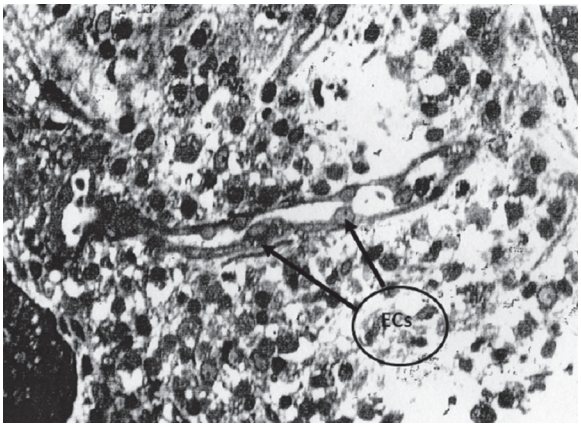

Figure 1. ECs protruding into vascular tumefaction. In some places the lumen is obstructed. 5.300×.

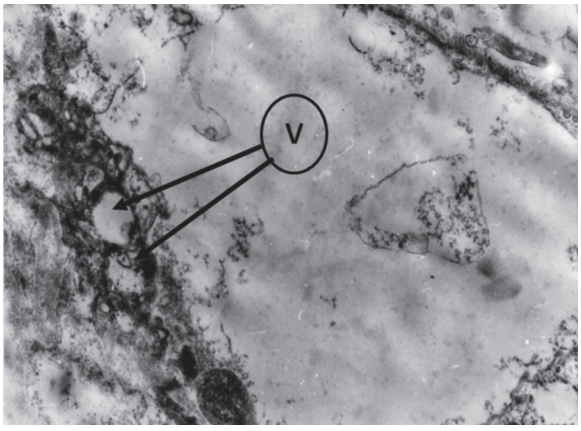

Figure 2. EC vacuolization in cytoplasm. 35.000×.

patients, with non bleeding varices. The esophageal biopsies from these alcoholic cirrhotic patients showed the presence of pronounced morphological alterations. The lower third of the esophagus showed submucosal capillary aneurysmatic vascular aspect, with hyperplasic endothelial cells, some of which presented necrotic features.

It is well established that cirrhotic patients with portal hypertension present several systemic and splanchnic circulatory derangements, including increased splanchnic blood pressure and formation of collateral veins. As described by Irisawa et al. [6], a serious complication is commonly present in this syndrome: the gastroesophageal variceal rupture and consequent bleeding.

Hashizume et al. [7], studied nine esophagogastric postmortem specimens from portal hypertensive patients, and described dilated intraepithelial channels and numerous small superficial collateral veins, in a longitudinal arrangement. Using portal hypertensive rats with partial portal vein ligation, Ichikawa et al. [8], described a swollen and hyperemic gastric mucosa, and reported that the endothelial cells from the mucosal microvessels had very prominent enlarged cytoplasm, and nucleus, producing a sort of sub obstruction of the capillary lumen.

It has been recognized that ECs behave as regulators of regional blood flow and vascular resistance [6], and as an

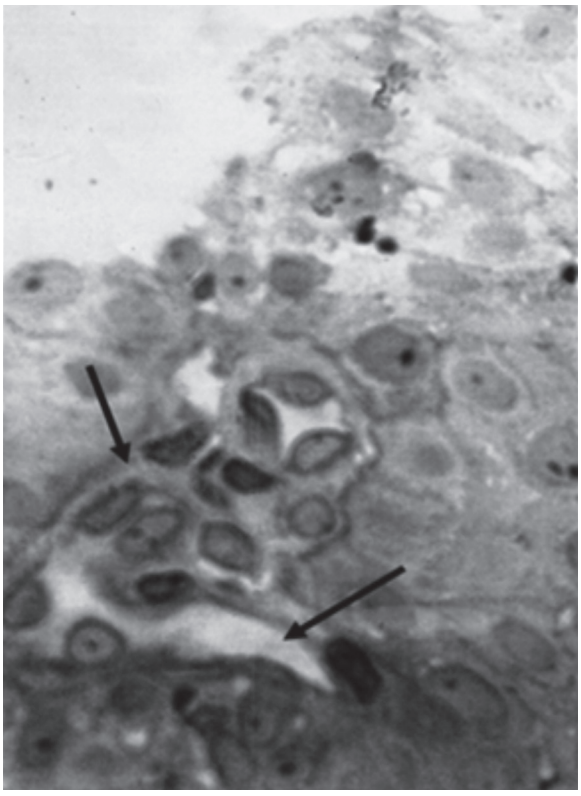

Figure 3. Esophageal mucosa and submucosa with disturbed morphology. Capillaries with aneurysmatic aspect, cells floating inside. Hematoxylin Eosin. 200×.

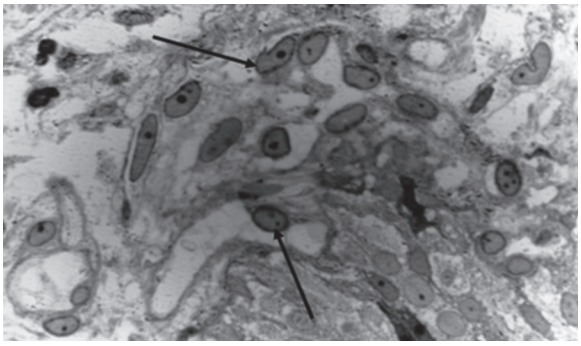

Figure 4. Same as Figure 3 but with higher magnification. A lacunar aspect with some cells floating, which are probably necrotic ECs. Hematoxylin Eosin. 400×.

important regulator of local vascular tone. It has been concluded that a dynamic vasoconstrictor-vasodilatator interaction controls tissue perfusion [9].

Increased portal pressure abnormally transmits its blood flow to abdominal thoracic collaterals and, the azygos vein; hence, pressure is exerted on esophageal veins, thus probably contributing probably to the development of varices. It can be presumed that the persistent high pressure exerted on this area is able to modify the morphology and concomitantly the function of its endothelial cells [10].

In cirrhosis, the hyperdynamic circulation in the splanchnic area, develops an increased blood flow and transmural

pressure, through collateral vessels, pushing the hypertensive portal circulation in direction to the lower pressure systemic venous circulation [11].

The ECs from the esophageal microcirculation, like its EC counterparts in other tissues, are able to synthesize vasoactive molecules [12]. The loss of these secretion substances, especially those like NO dependent action, prevails in the first step, and the vasoconstrictor substances, (ETs) enhance the portal hypertensive inflow pressure.

In the following step, the chronic pressure acts on the esophageal venous circulation, deteriorating the endothelial cellular existence itself.

Rockey et al. [13] have shown that, in the cirrhotic liver, both the enzymatic activity of nitric oxide synthase (NOS) and NO production decrease in liver sinusoids, thus leading to the predominance of vasoconstrictor substances (ETs) [14] hence intrahepatic vascular resistance, normal vasoregulation is damaged.

Endothelial cells are even removed from basal membrane by increased chronic portal pressure and separated from its matrix, as was documented in our investigation (Figures 3, 4).

Normally, the direction of venous blood flow in the horizontal esophageal veins from the submucosa is directed to the azygos. It has been suggested that in PH, the flow direction is inverted from the azigos to the submucosa and that permanent chronic increased blood portal pressure, can cause a dysfunction of the endothelial cells and circulating perturbation, which secondarily lead to irreversible injury. These alterations contribute to creating changes in local circulation, thus adding, an important factor to zonal epithelial cells, aggravated by EC budding into the microvascular lumen. As time progresses and PH persists and the local circulatory derangements worsen, new varices can be produced.

Therefore, a two step mechanism probably exists in varices formation in PH patients: a hyperdynamic circulation, due to portal hypertension, and the reversion of blood venous flow: azygos to esophageal mucosal veins and, as a consequence, the production of functional and morphological alterations due to local endothelial cell damage. The formation of small lacunar veins may represent the initial step of varices creation.

Hashizume et al. [7], described the venous conformation of the lower esophagus and stomach in portal hypertensive cadaveric specimens and found dilated intraepithelial channels and subepithelial superficial veins located over the esophageal varices, similar to our findings.

The presence of venous tortuosity and aneurysmatic aspect of esophageal veins, suggests that turbulence and irregular blood flow direction, can thus affect even more the venous wall, including its endothelial cells. Actually, this venous blood flow probably causes qualitative and quantitative alterations in its secretions. Several studies point to the importance of NO, which is produced by endothelial cells, in the modulation of capillary blood flow and its role in PH [9,14].

Several lines of evidence have suggested the existence of an increased synthesis of NO in experimental cirrhosis [1].

Nitric oxide plays an important role in the regulation of the blood flow and arterial pressure, and in normal circumstances, the inhibition of the endogenous NO system in experimental models and healthy subjects is associated with vasoconstriction and arterial hypertension.

According to Pinzani et al. [15], ET-1, a powerful vasoconstrictor, also secreted by endothelial cells, is over expressed in the cirrhotic liver, contributing to the genesis of varices and to maintaining the increased liver sinusoidal resistance. The low levels or absence of NO in tissues and the presence of ET-1 cause esophageal stress in the circulating tissue perfusion.

The NO released from endothelial cells, diffuses into the extracellular space and, activates the soluble guanylate cyclase of the underlying vascular smooth muscle cells. Through the generation of the messenger guanosine 3'-5' cyclic monophosphate, vascular relaxation takes place.

An increase in plasma endothelin has been described in cirrhotic patients by Bruno (16) and Salo et al. [17] and Uchibara et al. [5].

In prehepatic portal hypertensive rats, Tarnawski et al. [18] documented activation of the gastric gene for endothelial eNOS, localized in mucosal and submucosal vessels.

As shown in this experiment, the presence of severe morphological derangements in ECs from esophageal microcirculation induces important functional alterations in the production of vasoconstrictor, vasodilator secretions.

The esophageal endothelial dysfunction produced, by portal hypertensive chronic action; bring a profound alteration in its blood flow perfusion. This phenomenon contributes to weakening the esophageal mucosa and submucosa lining, facilitating the production and later ruptures of varices.

The development of port systemic collateral vessels connecting PH vasculature and the lower pressure of the systemic venous system is a further mechanism for the appearance of varices. Angiogenesis, dilatation, and increased blood flow through collateral vessels accompany collateral circulation.

After to explain the mechanism of the presence of varices in cirrhotic patients with PH there is a question to answer: which is the significance of the different endoscopic aspects of these esophageal varices, such as, the red spot tortuosities. Perhaps they evidence a way of development or varix evolution.

Portal hypertension is clearly involved in the formation and structure of esophageal varices with clear participation of the endothelial cell ultrastructure. According to previous and present studies, PH constitutes a major complication in developing a syndrome that involves regions of the central nervous system, including its blood-brain-barrier (BBB).

To reach these conclusions we used rat models of PH with normal livers and narrow portal vein [19–22]. We also used another rat model with the common bile duct ligation and another with PH after liver damage due to injection of toxic monocrotaline [23].

It must be recalled that BBB is formed by astrocyte processes and endothelial cells with tight junctions that are injured during PH models, increasing its permeability. With the disappearance of ligature and PH, BBB permeability returns to normal [24].

In human alcoholic cirrhosis with PH, systemic vascular responsiveness is altered, showing a resetting in the baroreflex function and alteration of the parasympatic nervous system [25,26].

Conclusions

It is possible that prolonged portal hypertension, through the inverted blood flow direction in esophageal perforating veins, causes anomalous local circulation. The important morphological alterations in endothelial cells from esophageal veins can be the expression of this anomalous circulation. Once endothelial cells are injured, it is possible that its vasoconstrictor and vasodilator secretions become modified or suppressed. Hence, their normal function controlling tissue perfusion fails, aggravating local tissue conditions. The pharmacological correction of PH may ameliorate the syndrome.

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© E&C Hepatology, 2011; 7(3-4): 16-19

Experimental & Clinical Hepatology

Received: 2011.09.09
Accepted: 2011.10.19
Published: 2011.12.15

Progress in the detection of productive HCV infection – the presence of the non-structural NS3 protein in peripheral blood mononuclear cells (PBMC)

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Background: The analysis of viral productive replication sites including cells of the immune system is important for the understanding of HCV pathogenesis.

The aim of the study was to search for the presence of NS3 HCV protein in PBMC with the simultaneous identification of the phenotype of the infected cell.

Material/Methods: PBMC were collected in the Warsaw Hospital of Infectious Diseases, from 17 HCV-infected and 17 uninfected subjects.

For the analysis of the nonstructural NS3 protein in PBMC, the immunocytochemical methods and flow cytometry were used. Monoclonal antibodies were used to analyse the HCV nonstructural protein NS3 in PBMC smears whereas flow cytometry was used to identify the phenotype of infected cells: CD3, CD14 and CD19.

Results: The immunohistochemical analysis of HCV NS3 protein presence in PBMC showed positive results in the cytoplasm of the infected cells. The frequency of HCV proteins detection was highest in monocyte $CD14^+$ (4–57%).

Conclusions: The results of the immunocytochemical and flow cytometry analysis of the PBMC for the presence of NS3 protein showed that cells of the immune system are independent sites of HCV productive infection.

Key words: hepatitis C virus (HCV) • HCV NS3 protein • PBMC • flow cytometry, immunohistochemistry

Full-text PDF: http://www.expclinhep.com/fulltxt.php?ICID=969164

Word count: 957
Tables: –
Figures: 2
References: 30

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Background

Until recently Hepatitis C virus (HCV) was considered to be a solely hepatotropic virus, however it can also replicate extrahepatically [1–4]. The presence negative strand HCV RNA was shown in PBMC, bone marrow and other cells and tissues [5–7]. Productive HCV infection can be defined as new virus particles release which includes all viral components including protein synthesis [2,4,6]. Detection of viral productive replications sites is essential for better understanding of HCV pathogenesis.

The consequences of the extrahepatic HCV replication is poorly understood due to the number of factors, both viral such as: virulence, infectious dose, adaptive strategies, as well as host factors, particularly condition of the immune system [8,9].

For the routine, clinical diagnosis of HCV infection the following techniques are used:
1. The serologic methods: detection the anti-HCV.
2. Molecular techniques: qualitative and quantitative PCRs (monitoring of antiviral therapy) [12–15].

Immonocytochemical and histochemical methods or flow cytometry may help to identify the phenotype of the cell productively infected with the virus [17,18]. The introduction of these methods are limited mostly by the technical difficulties, particularly non-specific reactions of antibodies with viral antigens, which cause of false positive results. The selection of appropriate antibodies, the development of the optimal conditions and appropriate control samples are necessary to obtain repetitive and reliable results [19,20].

The purpose of the study was to show the presence of the NS3 HCV protein in PBMC with the simultaneous identification of the phenotype of infected cell.

Material and Methods

Material

PBMC were collected in Warsaw Hospital of Infectious Diseases, from 17 HCV – infected and 17 uninfected controls.

Methods

To analyze nonstructural NS3 protein in PBMC, immunocytochemical methods and flow cytometry were used.

Detection of HCV NS3 in PBMC

PBMC smears preserved in acetone/chloroform were incubated with primary monoclonal anti-NS3 antibody (Novocastra Laboratories), following incubation with Alexa 488 goat anti-mouse secondary antibody (Molecular Probes). To stain the cell nuclei, preparations were incubated with Hoechst reagent and analyzed by using of Nikon 330–380 nm UV-2A filter.

The control material included liver biopsy samples from HCV-infected patients (positive samples) and uninfected patients (negative control). The specificity of the method was verified by an "internal" reaction control without a primary antibody.

Flow cytometry was used to identify the phenotype of the cells infected: CD3, CD 19 and CD14.

The detection of non-structural HCV NS3 protein in PBMC by means of flow cytometry.

Surface staining

PBMC subpopulations were phenotyped by flow cytometry using the following antibodies: CD3-PE, CD14-PE, CD19-PE and isotypic control IgG1-PE (Beckman Coulter). Cells were washed and incubated for 30 minutes with 5 µl antibody against surface antigens or appropriate isotype controls.

Finally, cells were fixed with 0.1 ml of 2% paraformaldehyde and 0.4 ml of PBS (Sigma) for 20 minutes and stored at 4°C until analysis (Beckman Coulter).

Intracellular staining

Fixed cells were permeabilized with Intra Prep (Beckman Coulter) and incubated with NCl-HCV-NS3 (Novocastra Laboratories) with Alexa Fluor 488 (Molecular Probes) antibodies or appropriate IgG 2b isotype controls (BD Biosciences) for 30 minutes at 4°C in the dark. After washing with 3 ml PBS, cells were resuspended in 2% paraformaldehyde until the analysis.

Results

The presence of NS3 HCV was shown in all PBMC smears from HCV-positive patients. The estimated number of the positive cells ranged from 4 to 10% (Figure 1).

The flow cytometry revealed NS3 positive cells in 5 patients: 4% to 57% – NS-positive CD 14^+ and 3% to 6% NS3-positive $CD19^+$ (Figure 2).

Discussion

It is known that non-structural HCV proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B) may interfere with cell proliferation, transformation and oncogenesis [9,14,19–21]. Thus detection of productive HCV infection is important for better understanding of the pathogenesis of infection.

The current diagnostics strategies of HCV infection requires a number of examination methods to be employed. For the characterisation of the productive viral replication, it is necessary to detect HCV-RNA and viral proteins [5,6,17].

The purpose of the work was to identify the presence of nonstructural NS3 HCV protein in PBMC from HCV infected patients and determine their phenotype. Development of immunocytochemical and flow cytometry methods and optimisation their sensitivity and specificity enabled to identify HCV non-structural proteins.

The immunocytochemical analysis of the PBMC smears for the presence of NS3 protein proved the productive infection with hepatitis C virus. The similar results we obtained for HCV NS3 expression in bone marrow [4,5].

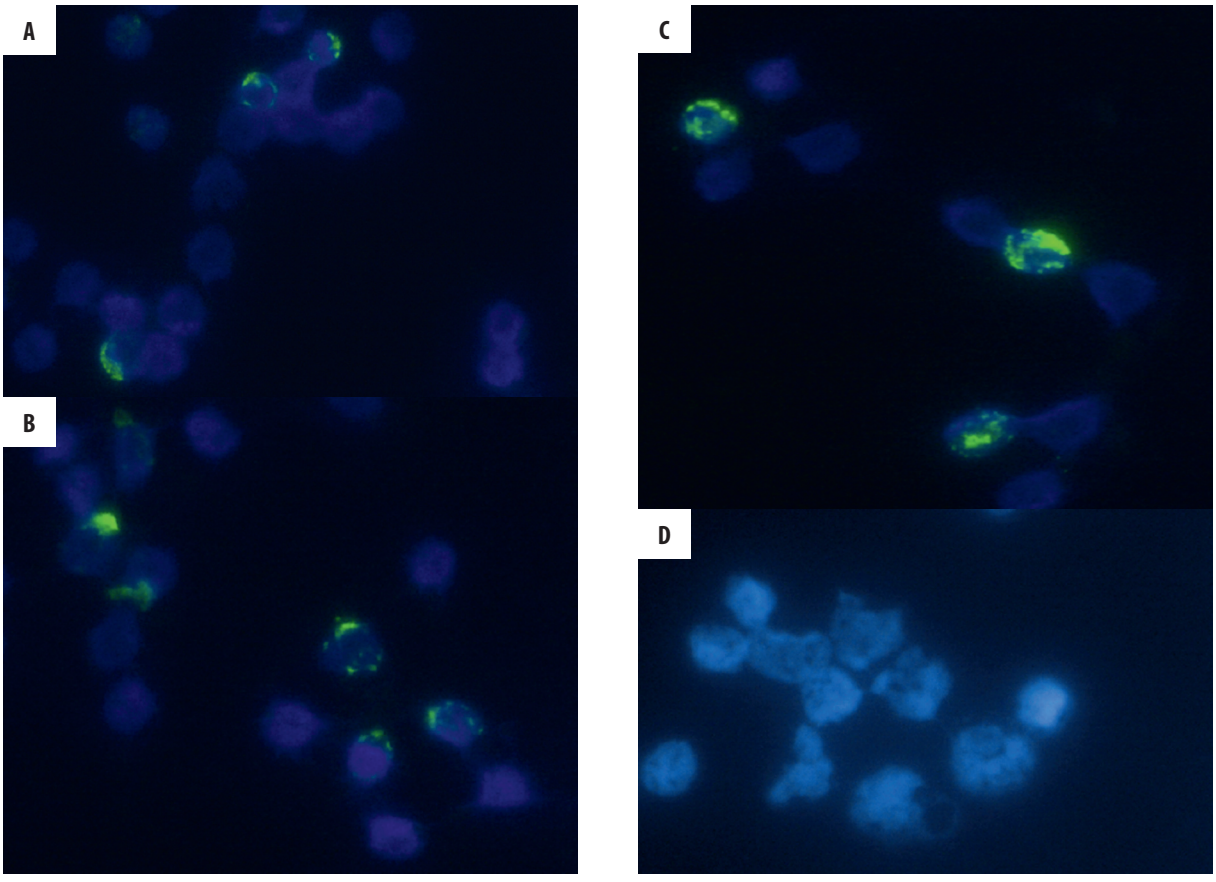

Figure 1. The presence of HCV NS3 protein in PBMC (**A–C**) positive results; (**D**) negative results.

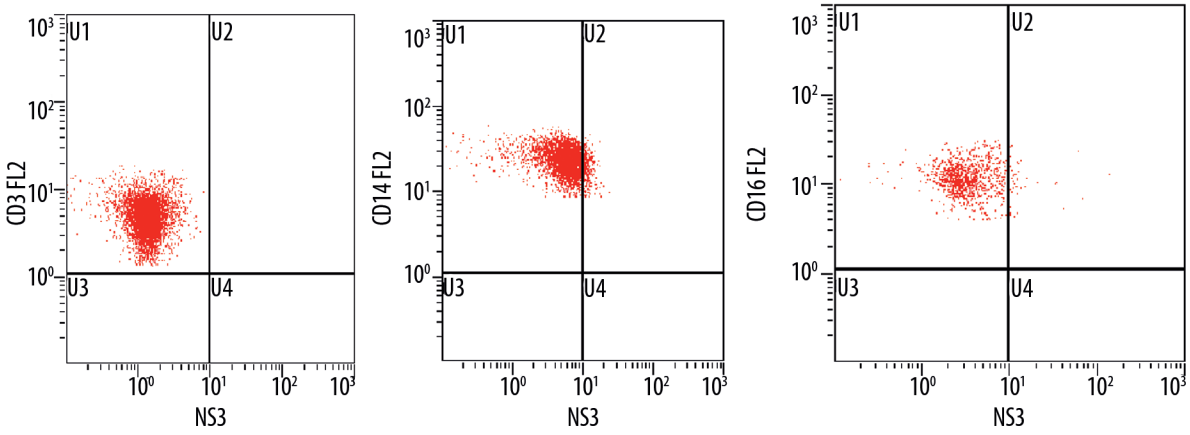

Figure 2. The presence of NS3 HCV protein in PBMC $CD3^+$ (negative results) $CD14^+$, $CD19^+$ (positive results).

The results of the immunocytochemical analysis and flow cytometry allow for the simultaneous detection of NS3 HCV and cell phenotype. Monocytes and CD 19^+ lymphocytes were found to be an essential site of the productive HCV infection [19–21]. The role of T-lymphocytes in HCV pathogenesis seems to be less important [15], however in earlier studies features of HCV replication were found in B and T cells as well as monocytes and macrophages [20,21].

On the other hand, the absence of NS3 HCV in $CD3^+$ lymphocytes may indicate that play a role in the latent phase of infection [14].

We showed that primary monocytes (CD 14^+) play the significant role as the place of the productive HCV infections.

T and B lymphocytes and monocytes/macrophages have been shown to be capable of sustained HCV replication [5,10,22–24]. To date, the clinical consequences of

macrophage HCV infection are not known, however it is known that disfunctional maturation of dendritic cells of monocyte origin is related to chronic HCV infection [2,5,25–30].

Further research in optimisation of the methods of HCV NS3 analysis in other cell populations may provide useful interactions concerning viruses-cell interactions.

Conclusions

The application of immunocytochemistry and flow cytometry in detecting the presence of NS3 HCV allows to identify the population of the cells productively infected with HCV. This indicates that cells of the immune system are independent sites of HCV replication.

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Experimental & Clinical Hepatology

Received: 2011.05.30
Accepted: 2011.06.26
Published: 2011.12.15

Effect of overexpression of metalloproteinases on TIMP-1 and procollagen IV gene expression in murine liver

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Background: Matrix metalloproteinases (MMP) play a crucial role in hepatic tissue remodeling. We investigated whether increased MMP expression in murine liver mediated by adenoviral gene transfer affects the expression of other genes involved in the hepatic extracellular matrix metabolism.

Material/Methods: MMP-2, 3 and 9 cDNA using wild type (wt) as well as mutant (mut) sequences without protease activity was constructed and cloned into adenoviral expression vectors. Quantitative real time RT-PCR assays were established to determine tissue inhibitor of metalloproteases (TIMP)-1 and procollagen IV (proCOL IV) levels after adenoviral MMP overexpression in murine liver.

Results: Overexpression of both wt and mut MMP *in vivo* caused increased TIMP-1 mRNA expression (10-fold). This effect was independent from catalytic MMP activity. In contrast, expression of proCOL IV mRNA decreased slightly after overexpression of MMP-2 wt/mut, whereas inactive MMP-3 and MMP-9 decreases COL IV expression up to 10-fold.

Conclusions: The regulation of genes involved in the hepatic ECM metabolism like TIMP-1 and proCOL IV may be affected by an upregulated hepatic MMP expression independent from enzymatic MMP activity.

Key words: **extracellular matrix • fibrosis • matrixmetalloproteinase**

Full-text PDF: http://www.expclinhep.com/fulltxt.php?ICID=969167

Word count: 2878
Tables: 2
Figures: 3
References: 18

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Background

Hepatic fibrosis is the hallmark of chronic liver diseases. It is a process based on imbalanced matrix degradation and synthesis which ultimately generates an accumulation of matrix proteins like collagens, laminin, hyaluronan and fibronectin within the liver [1]. Extracellular matrix (ECM) turnover and remodeling are centrally controlled by matrix metalloproteases (MMP) and their specific inhibitors (TIMP).

MMP are a family of zinc-dependent proteases secreted in latent zymogen form. Interactions between cysteine residues in the propeptide domain and the zinc atom located in the catalytic site of all MMP are responsible for maintaining the protease in an inactive state. MMP activation can occur *in vitro* or *in vivo* by cleavage of the propeptide by proteases or by disruption of the zinc-cysteine bond [2,3]. MMP have been demonstrated playing a crucial role in ECM remodeling during fibrotic processes in liver.

To date, 22 different members of the protein family are known [4], but only a few of them have been identified to be of major importance in liver, including MMP-1, MMP-2, MMP-3, MMP-9 and MMP-14 [5]. Accumulation of ECM proteins results from both increased synthesis and decreased degradation by MMP [5]. The decreased activity of MMP in liver fibrosis is mainly due to an increased expression of their specific inhibitors, tissue inhibitors of metalloproteinases (TIMP).

The role of TIMP is not only to block the protease activity, but to modulate MMP function. TIMP-1 binds to and inhibits activated collagenases. This leads to protection of newly synthesized collagen from immediate degradation by MMP. Furthermore, TIMP-1 is able to prevent activation of pro-MMP [6]. In chronic liver disease, significant increases in TIMP-1 and TIMP-2 expression have been observed, thus preventing the degradation of collagen [7,8].

The currently available data suggest that MMP and TIMP play a key role in liver fibrosis, but it is unknown whether MMP – beyond enzymatic matrix degradation – have effects on the regulation of TIMP and matrix protein expression in the liver.

In the present study, we investigated whether increased levels of MMP-2, MMP-3 and MMP-9 in the liver will influence tissue inhibitor of metalloproteases (TIMP)-1 and procollagen IV (proCOL IV) gene expression.

Material and Methods

Generation of adenoviral recombinants

Full-length cDNA of wt-MMP-2, wt-MMP-3 and wt-MMP-9 was cloned by RT-PCR after RNA isolation from mouse liver samples. Inactive mutants of MMP-2 (a1508c and g1509t), MMP-3 (a738c) and MMP-9 (a1211c) were generated by *site directed mutagenesis* PCR (Stratagene). This leads to a change of the amino acid glutamate to alanine within the zinc-binding motive of the protease-domain. Correctness of all cloned genes was confirmed by sequence analyses.

The adenoviral vector system was kindly provided by Dr. Bert Vogelstein (John Hopkins Oncology Center, Baltimore, USA). The vector pAdtrack-CMV expressing the green fluorescent protein (GFP) has been described elsewhere [9]. For generation of different adenoviruses expressing the MMP-2, MMP-3 and MMP-9 derivatives, full-length mouse cDNA was inserted into the pAdTrack-CMV shuttle vector. Correct ligations were confirmed by PCR analysis and sequencing. The recombinant plasmids were linearized with Pme I and cotransformed into *E. coli* BJ5183 containing the pAdEasy-1 backbone plasmid (Stratagene). The generation of recombinant adenoviral plasmids was performed by homologous recombination in *E. coli* as described elsewhere [9]. After confirmation of recombination by PCR and restriction endonuclease digestion, adenoviral vector DNA, digested with Pac I and ethanol precipitated, was transfected into the adenovirus-packaging Human Embryonal Kidney cell line (HEK 293) (DSMZ, Braunschweig, Germany). Transfected cells were monitored for GFP expression and harvested 7 days after transfection. After generation of high titer viral stocks, viruses were purified by double CsCl density gradient ultracentrifugation. Particle concentration was determined by OD_{260} reading. Plaque forming units (pfu/ml) were determined by standard plaque assay on HEK 293 cells.

Cell culture

HEK 293 cells were grown as monolayers on plastic dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 1U/ml penicillin and 100 µg/ml streptomycin.

Viral infection

Confluent grown HEK 293 cells were transfected using a virus concentration of 1×10^8 pfu/ml in DMEM containing 2% FCS, 100 µg/ml streptromycin and 1U/ml penicillin. After 48h cells were centrifuged and harvested by 3 cycles of freezing/thawing. Protein concentration was determined by immunturbidimetry using benzethoniumchloride.

Protein analysis

Western-blot analysis was performed by immunodetection of MMP-2, MMP-3 and MMP-9 in cell extracts using goat anti-mouse MMP-2 IgG, goat anti-mouse MMP-3 IgG and goat anti-mouse MMP-9 IgG antibodies (Sigma-Aldrich, Steinheim, Germany). Primary antibodies were diluted 1:10,000. For visualization peroxidase-conjugated sheep anti-goat IgG secondary antibodies (Sigma-Aldrich) were diluted 1:20,000.

Proteolytic activity of recombinant wild-type MMP and proteolytic inactivity of recombinant MMP mutants were confirmed by zymography [10]. 1 mg/mL gelatin was copolymerized in a 10% PAA gel. 5 µg total protein extract were loaded for gel electrophoresis.

Animal model/application of adenoviruses

Adenoviruses were dialyzed against native buffer containing 10 mM Tris/HCL pH8.0, 10 mM $MgCl_2$ and 50 mM NaCl prior to infection of animals. 1.5×10^9 pfu of virus were injected into the tail venes of 8 weeks old balb/c mice (16–20 g). After sacrifice, livers were explanted and stored at –80°C in RNA-later buffer (Roche Diagnostics, Mannheim, Germany). Animal studies were conducted

according to the national animal welfare regulations and approved by the local Animal Care and Use Committee of Niedersächsisches Landesamt and the Hannover Medical School (No. 33.9-42502-04-07/1373).

RNA isolation and generation of standard curve

mRNA from liver tissue was isolated using the Blood/Bone Marrow mRNA Isolation System® (Roche Diagnostics, Mannheim, Germany) according to the suppliers instructions.

The RNA concentration was determined spectrophotometrically using the RiboGreenTM RNA Quantitation Kit (MoBiTec, Göttingen, Germany) on a F2000 fluorescence photometer (Hitachi). First-strand cDNA was synthesized from 50 ng mRNA, using Superscript II reverse transcriptase (Invitrogen, Karlsruhe, Germany) and 250 ng random primer (MWG Biotech, Ebersberg, Germany).

The following cDNA sequences were chosen to amplify cDNA fragments, which were cloned into the pBluescript KS^+ vector (Stratagene, La Jolla, USA). The according accession numbers of the NCBI reference sequences are given in parentheses:

MMP-2 (NM_008610: nucl. 298-2287), MMP-3 (NM_010809: nucl. 75-1513), MMP-9 (NM_013599: nucl. 7-2199), GAPDH (NM_008084: nucl. 317-674), TIMP-1 (NM_001044384: nucl. 195-743), TIMP-2 (NM_011594: nucl. 321-980), procollagen IV α1 (NM_009931: nucl. 3828-5367), SMA (NM_007392: nucl. 423-729).

Recombinant plasmid DNA (1 µg) was linearized by Asp718 digestion (New England Biolabs, Frankfurt, Germany) prior to the *in vitro* transcription of RNA using the RNA Transcription Kit (Stratagene). DNA was digested by treatment with DNase I and Proteinase K. After phenol/chloroform treatment and ethanol precipitation, RNA was reconstituted in RNase free water according to manufacturer's instructions.

Molecule numbers were determined under consideration of the molecular weight and Avogadro's number. Stock solutions of *in vitro* transcribed RNA containing 10^{10} copies/µl were prepared and stored at –80°C. For subsequent dilution a solution containing 250 µg/l tRNA (Roche Diagnostics) was used. Each mRNA standard was checked by RT-PCR with corresponding primers and hybridization probes to avoid genomic DNA contamination.

Quantitative real-time RT-PCR

Quantitative real-time RT-PCR was performed using the QuantiTect Kit (Qiagen, Hilden, Germany) and the LightCycler instrument (Roche Molecular Biochemicals, Mannheim, Germany). All primers and hybridization probes were received from MWG Biotech (Ebersberg, Germany). Sequences of the primers and hybridization probes are listed in Table 1. Briefly, 5 µl cDNA were mixed with 15 µl master mix containing forward-primer (10 pmol), reverse-primer (10 pmol), donor hybridization probe (4 pmol), acceptor hybridization probe (4 pmol), 1 µl DMSO and 10 µl QuantiTect Mix. H_2O instead of cDNA served as negative control. Each PCR included a standard and a no template control. The quality of cDNA for each run was estimated by quantification of glycerealdehy-3-phosphate dehydrogenase (GAPDH). The gene copy number was calculated from serially diluted plasmids (5×10^2 to 5×10^7 RNA equivalents).

Table 1. Primers and hybridization probes for real-time PCR assays.

	Amplification primers and hybridization probes (5′-3′)
MMP-2	Sense: TCC GTG GTG AGA TCT TCT TCT Antisense: GTA CTC ATT CCC TGC GAA GAA C Hp donor: CCT GAG CTC CCA GAA AAG ATT GAC GCT GT Hp acceptor: ATG AGG CCC CAC AGG AGG AGA AGG
MMP-3	Sense: GCT GAA CGA TGG ACA GAG Antisense: CTT GGC TGA GTG GTA GAG T Hp donor: 5′- Quencher- TTC CTG GTT GCT GCT C-3′-FITC
MMP-9	Sense: TCA TCC AGT TTG GTG TCG C Antisense: CCT CGA AGG TGA AGG GAA AG Hp donor: GTT CAG GGA GAT GCC CAT TTC GAC GA Hp acceptor: GAC GAG TTG TGG TCG CTG GGC AAA GG
TIMP-1	Sense: CAG ATA TCC GGT ACG CCT ACA C Antisense: GAA GCT GCA GGC ACT GAT G Hp donor: CAA GTC CCA GAA CCG CAG TGA AGA GTT TC Hp acceptor: ATC ACG GGC CGC CTA AGG AAC G
Procollagen IV α1	Sense: CCT GGC CAG AAA GGA GAG A Antisense: CCA TGG TAA AGA ATT TTG GTC C Hp donor: CCC ATC TGT TGA CCA CGG CTT CCT TGT GA Hp acceptor: CAG GCA TAG TCA GAC AAT AGA TGA CCC AC
GAPDH	Sense: TGC TGA GTA TGT CGT GGA GTC Antisense: GGA TGC AGG GAT GAT GTT CT Hp donor: GAC AAC TTT GGT ATC GTG GAA GGA CTC ATG ACCACA Hp acceptor: CCA TGC CAT CAC TGC CAC CCA GAA GAC T
SMA	Sense: GGG AGA AAA TGA CCC AGA TTA TG Antisense: TAG CAC AGC TTC TCC TTG ATG TC Hp donor: CGA GAT CTC ACC GAC TAC CTC ATG AAG ATC CT Hp acceptor CTG AGC GTG GCT ATT CCT TCG TGA CTA CTG

Hybridization probes were labeled with 3′-Fluorescein (donor) and 5′-Cy5.5 / 3′-phosphate (acceptor) except for MMP-3.

Evaluation of the RT-PCR assays

To provide precision data, RNA pools from mouse liver preparations were assayed on eight consecutive days (n=8) and the RNA equivalents of the transcripts per ng total mRNA were calculated. Mean values, standard deviations and coefficients of variation (CV%) were calculated.

Statistical analysis

Data were analyzed with the statistical software package SPSS 13.0 for Windows. Student's two-tailed t-tests were performed to calculate statistical differences between the results of the different variables. P-values of less than 0.05 were considered statistically significant.

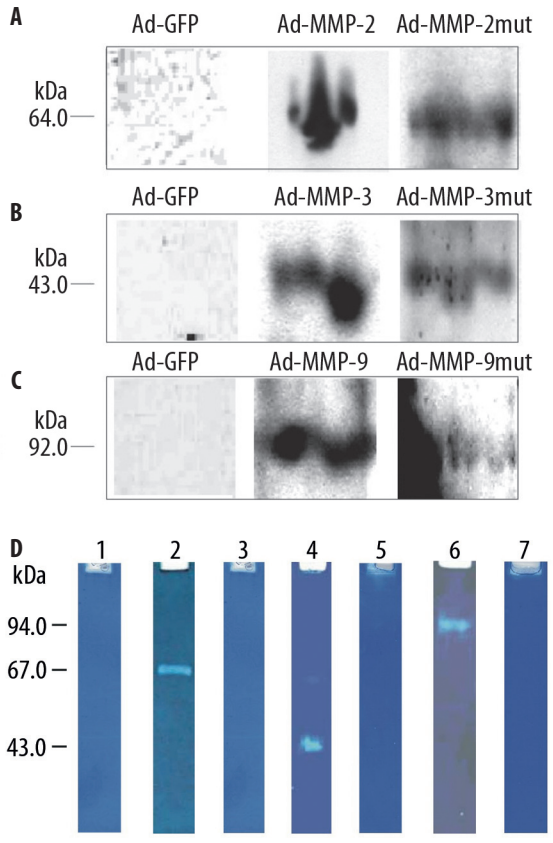

Figure 1. *In vitro* characterization of recombinant adenoviruses. (**A–C**) Protein expression of wt/mut-MMP-2, wt/mut-MMP-3, wt/mut-MMP-9 by HEK 293 cells after adenoviral transduction. Total cell lysates were analyzed by Western blotting using anti-MMP-2, anti-MMP-3 and anti-MMP-9 antibodies. GFP adenovirus was used as negative control. (**D**) Proteolytic inactivity of the MMP-2, MMP-3, MMP-9 mutants in lanes 3, 5, 7 was shown in comparison to the wild-type MMP in lanes 2, 4, 6 by gelatine zymography. GFP adenovirus was used as negative control (lane 1).

Results

Generation and characterization of recombinant adenoviruses

Several Ad5-derivatives expressing proteolytically active and inactive MMP-2, MMP-3 and MMP-9 were generated by using the Adeasy adenoviral vector system. Full-length cDNA of MMP-2, MMP-3 and MMP-9 were cloned into the pAdTrack shuttle-vector. The inactive mutants of MMP-2 (E402A), MMP-3 (E219A) and MMP-9 (E404A) were generated using *site directed mutagenesis* PCR. All cloned constructs were confirmed by sequence-analysis. After homologue recombination with the virus-backbone cDNA viruses were amplified in HEK 293 cells.

After purification on a CsCl gradient, the viral titer was determined by standard plaque assay ranging from 1×10^{11} to 1×10^{12} pfu. Similarly, a high-titer viral preparation was obtained for adenovirus expressing only GFP, which was used as a control virus in all experiments.

Table 2. Intra-assay and inter-assay variation of RT-PCR analyses for MMP-2, MMP-3, MMP-9, TIMP-1, Procollagen IV and GAPDH.

	Intra-assay (n=8)	Inter-assay (n=8)
MMP-2	$1.3\times10^5\pm1.7\times10^4$ (13)	$1.5\times10^4\pm2.4\times10^3$ (16)
MMP-3	$4.8\times10^4\pm7.1\times10^3$ (15)	$4.1\times10^4\pm7.8\times10^3$ (19)
MMP-9	$3.9\times10^3\pm4.0\times10^2$ (10)	$1.1\times10^4\pm5.1\times10^3$ (46)
TIMP-1	$2.0\times10^2\pm2.3\times10^1$ (12)	$3.4\times10^2\pm1.5\times10^2$ (44)
Procollagen IV	$1.3\times10^5\pm7.9\times10^4$ (61)	$1.1\times10^5\pm1.4\times10^5$ (127)
GAPDH	$1.1\times10^7\pm9.9\times10^5$ (9)	$1.2\times10^7\pm1.4\times10^6$ (12)

Shown are mean values of molecules/ng RNA ±SD. Coefficients of variation for the individual assays in% are shown in parentheses.

Adenoviral viruses were checked for functionality *in vitro* using HEK 293 cells. After adenoviral injection, protein expression was verified by SDS-PAGE and Western blot analysis of cell culture media. As shown in (Figure 1A–C), expression of MMP-2, MMP-3 and MMP-9 was detected as a 64 kDa, 43 kDa and 92 kDa protein both for active and inactive variants in concentrated cell supernatants of treated cells. Before the viruses were used in mice, the proteolytic properties of all constructs were confirmed. The complete loss of proteolytic activity of the MMP mutants was tested in comparison to wt-MMP using protein extracts for gelatin zymography. Only wt-MMP-2, wt-MMP-3 and wt-MMP-9 were able to degrade gelatin and to produce the characteristic degradation bands of 64 kDa, 43 kDa and 92 kDa (Figure 1D). There was no proteolytic activity detectable for the mutants and the GFP-control.

Quantitative real-time RT-PCR assays

For the quantification of MMP-2, MMP-3, MMP-9, TIMP-1 and procollagen IV mRNA expression, quantitative real-time RT-PCR assays were established. Inter-assay and Intra-assay variation was assessed by using cDNA measured 8 times within and between run. The evaluation data are listed in Table 2.

Highly efficient liver transduction by adenoviral gene transfer

We examined the efficacy of the adenoviral gene transfer in our animal model after tail vein injection of 1.5×10^9 pfu Ad-GFP. Similarly to earlier results [11,12], in liver of balb/c mice GFP expression was observed in more than 50% of hepatocytes (data not shown).

To determine the effect of virus application on the expression of MMP-2, MMP-3, MMP-9, TIMP-1, proCOL IV and GAPDH, the Ad-GFP control virus was used. Before and after 3, 10 and 21 days after injection liver samples were analyzed by quantitative real-time RT-PCR. No significant differences were found for MMP-2, MMP-3, MMP-9, TIMP-1, proCOL IV and GAPDH mRNA expression compared to the timepoint before virus application. Therefore, the virus itself has no significant effect on mRNA expression of the matrix-associated genes.

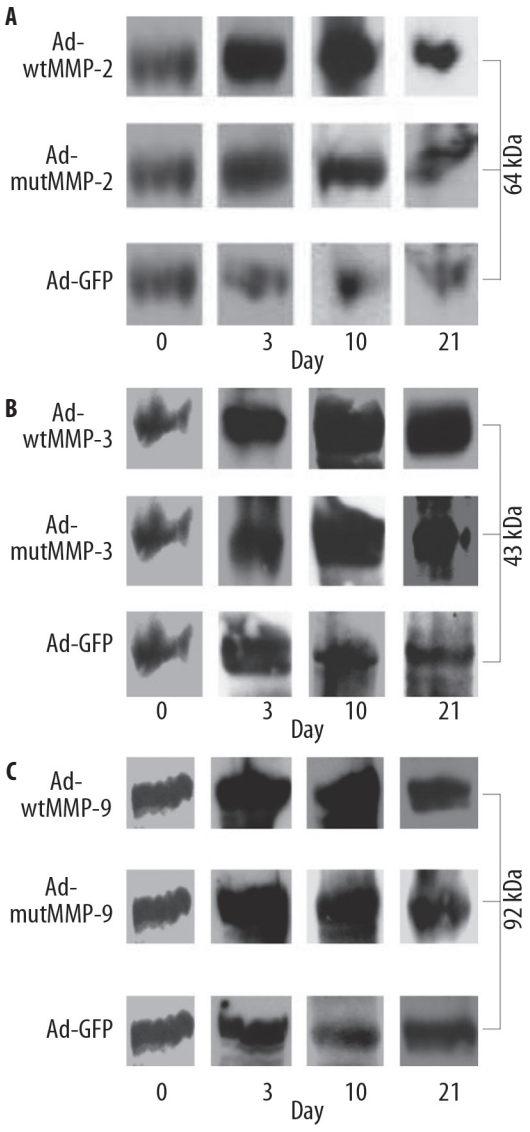

Figure 2. *In vivo* overexpression of wild-type and mutant MMP in liver tissue after adenoviral transfection was analyzed by Western blotting. Hepatic protein extracts were analyzed for (**A**) MMP-2, (**B**) MMP-3 and (**C**) MMP-9 expression after 0, 3, 10 and 21 days p.i.

Intrahepatic expression efficacy of MMP-2, MMP-3 and MMP-9 after adenoviral infection was analyzed by Western blot analyses (Figure 2). Mice were treated with a single injection of 1.5×10^9 pfu via tail vein injection. Livers were harvested 0, 3, 10 and 21 days after injection. The protein extracts were analyzed by Western blot analysis. The maximum of expression was detected 3–10 days after injection, whereas MMP-3 overexpression was detectable up to 21 days after transduction, respectively (Figure 2).

Effect of Ad-MMP gene transfer on TIMP-1 and proCOL IV mRNA expression

To study the effect of changes in hepatic expression of matrix associated genes after Ad-wtMMP-2, Ad-mutMMP-2, Ad-wtMMP-3, Ad-mutMMP-3, Ad-wtMMP-9 and Ad-mutMMP-9 transduction in mouse liver, four mice per MMP and timepoint were treated with a single injection of 1.5×10^9 pfu via tail vein. To exclude viral effects, all results were referred to a Ad-GFP control group. Livers were harvested after 0, 3, 10 and 21 days after viral infection and mRNA was isolated. The mRNA expression of the target genes TIMP-1, proCOL IV and GAPDH was analyzed by quantitative real-time RT-PCR and for each timepoint the mRNA expression after viral injection was compared to control mice (Ad-GFP) by statistical t-test.

In the GAPDH control group small but significant changes in mRNA expression were detectable 10 and 21 days after infection (Figure 3A–C). These changes were seen for all MMP overexpressed and they were independent from proteolytic MMP activity.

For TIMP-1, an increase of mRNA concentration was detectable after MMP overexpression (Figure 3D–F). In control mice exposed to Ad-GFP alone, no significant changes in TIMP-1 mRNA concentration were detectable. The highest effect on TIMP-1 mRNA expression was seen for hepatic MMP-9 overexpression (10-fold), but was also visible for MMP-2 and MMP-3 overexpression. There were no differences regarding to the wild-type or mutant forms of MMP indicating that the MMP-induced increase in TIMP-1 mRNA expression is independent from proteolytic MMP activity. Upregulation of TIMP-1 mRNA expression was detectable up to 21 days after virus application (Figure 3D–F).

A contrary effect was detected for proCOL IV mRNA expression (Figure 3G–I). Here, hepatic overexpression of MMP-2, MMP-3 and MMP-9 induced a significant decrease in proCOL IV mRNA expression with a minimal expression rate on day 10 *post infectionem* (p.i.) for MMP-2 (5–8 fold). This effect was detectable for both wild-type and mutant MMP-2 and MMP-9. This effect differed between MMP. For example, overexpression of mutant MMP-3 decreases the proCOL IV mRNA expression approximately 10-fold after 10 and 21 days p.i., but with wt-MMP-3 no significant changes in proCOL IV mRNA expression were detectable (Figure 3H).

Discussion

The main focus of MMP research has been on the modulation of tumor invasiveness and angiogenesis and to a lesser extent on their role in chronic liver disease and other fibrotic diseases. For more insight into MMP interaction with matrix associated genes, we generated adenoviral constructs coding for wildtype (wt) and proteolytically inactive (mut) MMP-2, MMP-3 and MMP-9. Functionality of these constructs was confirmed *in vitro*. Furthermore, efficient transfer of the adenoviral genes was shown in an *in vivo* animal model.

TIMP-1 is known for a central role in hepatic fibrogenesis. We therefore chosed TIMP-1 as a target gene. Making use of the natural tropism of adenoviral vectors to liver tissue, we were able to demonstrate a direct effect of overexpressed MMP on TIMP gene expression. As an example, TIMP-1 mRNA expression increased up to 10-fold after MMP-9 overexpression. The increase was independent from any catalytic activity of the MMP used. For MMP-2 and MMP-3 the effect was even higher for the mutant enzyme (8–10 fold) compared to the active enzyme (2–5 fold).

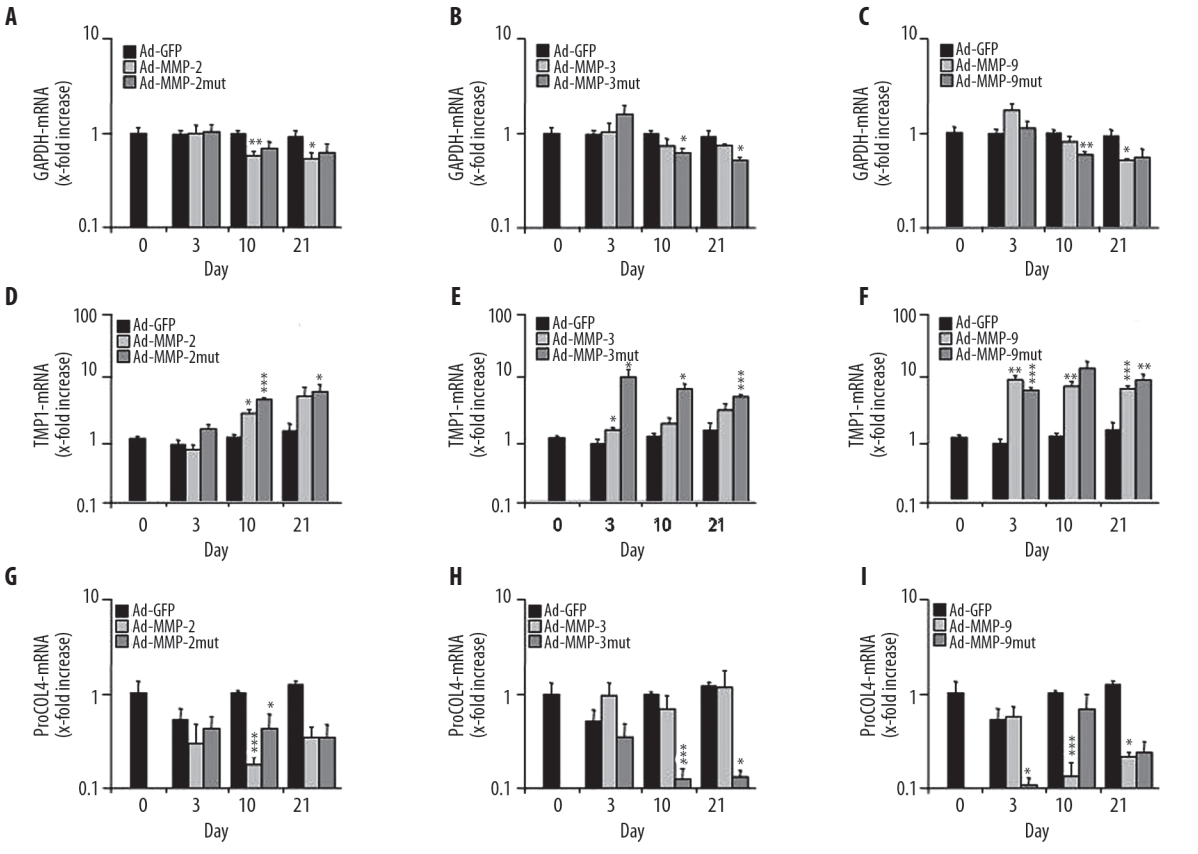

Figure 3. Quantitative real-time RT-PCR of mRNA expression of GAPDH (**A–C**), TIMP-1 (**D–F**) and Procollagen IV (**G–I**) in mouse liver after 0, 3, 10 and 21 days p.i. The mRNA concentration is presented proportional to control animals (Ad-GFP). The mRNA expression on each day after adenoviral transduction was compared between control animals and animals with MMP-overexpression by T-test (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

The demonstrated effects resemble a general behaviour in tissue biology, where increases in proteases are often paralleled by an increase of inhibitor molecules. Potential cellular sources of TIMP-1 increased expression are hepatic stellate cells as well as surrounding cells. Similar effects have been shown for the coagulation cascade and can also be seen in the liver, during chronic inflammation [13].

For the interaction between MMP and TIMP expression it also needs to be taken into account that MMP-9 and MMP-2 are involved in the activation of latent TGF [14], which regulates TIMP-1 expression. However, here we have clear evidence that catalytic MMP activity is not necessary to influence the expression of TIMP-1.

In contrast to our results, another study reports no effect of wildtype and mutant MMP-9 on TIMP-1 mRNA expression [15]. These investigations were carried out in a mouse model after CCl_4 injury, which renders the comparison of these data of limited value.

The regulation of TIMP-1 mRNA expression is directly associated with TGFβ, but it has also been shown that TIMP-1 is upregulated by several inflammatory cytokines like IL-1β, IL-6 and TNFα [16,17]. These pathways due to inflammation caused by virus application appear unlikely, because no effect on TIMP-1 expression was detectable after application of Ad-GFP control virus in our experiments. It can be speculated, if other domains than the protease domain are involved in TGFβ activation. Alternatively, other pathways without need for cleavage of latent signal molecules by MMP as the first step, may be involved.

The presented data illustrate the complex MMP/TIMP interaction in liver biology. Further experimentation will be required to elucidate the mechanisms underlying these associations.

Unlike most collagens, type IV collagen occurs only in the basement membrane. In the liver, it is deposited within perisinusoidal space (Dissé space), in between the endothelial sinusoidal cells and the vascular face of the hepatocytes. Type IV collagen has been shown to be involved in the development of hepatic fibrosis and cirrhosis. The remodeling is executed by MMP-2 and MMP-9 [18]. So far, there have been no reports showing an interaction between MMP expression and collagen IV expression in liver. We detected a significant decrease of procollagen IV mRNA expression independent from catalytic activity of MMP-2 and MMP-9. Similar data have been reported for type I collagen. In a mouse model, adenoviral overexpression of MMP-9 was able to reduce hepatic type I collagen [15].

Conclusions

In conclusion, our study shows that overexpression of MMP-2, MMP-3 and MMP-9 by adenoviral gene transfer results in a marked increase of TIMP-1 expression in liver, independent from catalytic activity of the overexpressed MMP. This may be interpreted as a functional control of overshooting MMP activity. In contrast, higher levels of MMP-2 and MMP-9 induced a reduction in proCOL IV gene expression, thereby promoting the basal membrane degrading activity of gelatinases.

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© E&C Hepatology, 2011; 7(3-4): 29-31

Experimental & Clinical Hepatology

Received: 2011.11.12
Accepted: 2011.12.06
Published: 2011.12.15

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

Atypical CT scan findings in a case of Wilson's Disease

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Summary

Wilson disease (WD) is an autosomal recessive disease of a membranebound copper-transporting ATPase. Clinical manifewstations are caused by deposition of copper on brain and liver causing different clinical manifestations and radiologic findings. We report a case with atypical finding on CT scan of brain which showed presence of bilateral symmetrical hypodensity at thalamo-capsular and frontal areas,not found commonly in WD along with minimal involvement of the lentiform nucleus.

Key words: **Wilson's disease (WD) • computed tomography • hypodensity**

Full-text PDF: http://www.expclinhep.com/fulltxt.php?ICID=970275

Word count: 691
Tables: –
Figures: 3
References: 5

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Background

Wilson's disease (WD) is an autosomal recessive disease caused by mutations in the ATP7B gene, a membrane-bound copper-transporting ATPase. Clinical manifestations are caused by copper deposition in the basal ganglia of the brain and in the liver. Various neurological and psychiatric symptoms may occur as well as one of three major hepatic disorders such as cirrhosis, chronic hepatitis or fulminant hepatic failure. The frequency of WD in most populations is about 1 in 30,000–40,000 [1].

Case Report

A 16 year old female presented to the ER with chief complaints of one day multiple episodes of generalised tonic clonic seizures with loss of consciousness in the in-between two successive convulsions. Each episode of convulsion lasted for about one minute. She was diagnosed as Wilson's disease (WD) five years ago when she presented with neuropsychiatric manifestations with a past history of jaundice. There was no history of fever with rash. She was on D-penicillamine for four years. However, for last one year since patient was asymptomatic she stopped taking medications. Her perinatal history and developmental milestones were normal. On examination (after seizures became controllable) she was drowsy but conscious, had vitals stable with bilaterally reacting pupils and bilateral Babinsky's sign positive, blood pressure 120/80 mm Hg. All the systems were within normal limits including nervous system and gastrointestinal system. Kayser-Fleischer ring was found on slit lamp examination (Figure 1). She was stabilised and her seizures were controlled by lorazepam IV followed by phenytoin IV. Laboratory investigations revealed: Hb 10.7 gm%; TC=1500/cc, platelets=200,000/mm^3; urea=36 mg/dL;Cr=0.8 mg/dL; Electrolytes in serum and liver function test were normal.CSF studies including electrophoresis were also found normal. HIV ELISA(1&2) was non-reactive. 24-hour urinary copper=82.06 ug/L and 139.50 ug/24 hours, serum ceruloplasmin was 15 gm/dL.Ultrasound of abdomen revealed coarse texture of the liver. Upper gastrointestinal endoscopy was normal.

The non contrast and contrast enhanced CT scan findings of brain showed bilateral symmetrical hypodense non enhancing areas involving thalami and lentiform nuclei having oblong much more hypodense zone at lateral putamina producing no mass effect. Also there were almost symmetrical hypodense nonenhancing areas involving bilateral medial frontal subcortical and deep white matter without any mass effect. Third and lateral ventricles are mildly dilated bilaterally. The impression was of symmetrical deep gray matter

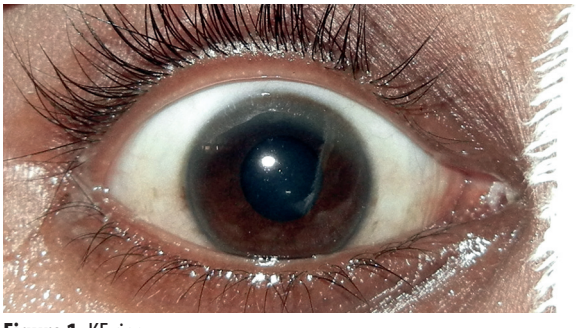

Figure 1. KF ring.

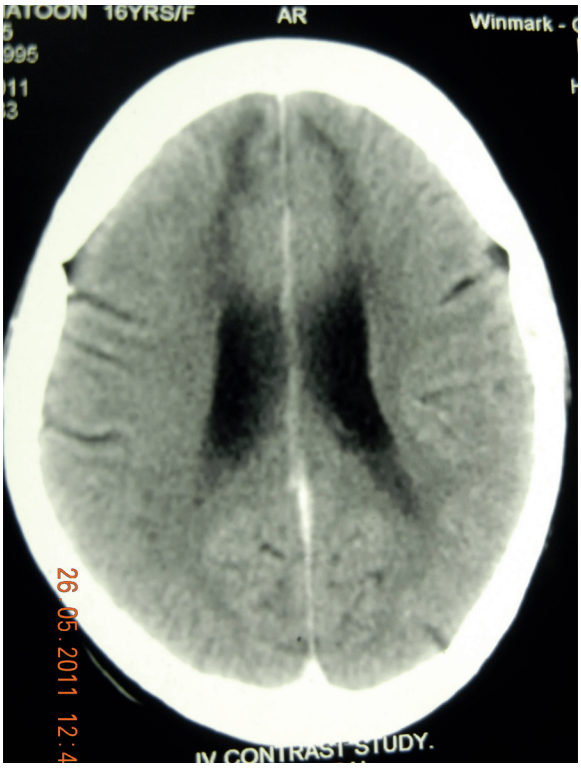

Figure 2. Contrast enhanced CT scan of Brain bilateral symmetrical hypodense non enhancing areas involving thalami and lentiform nuclei.

and white matter (frontal) involvement (Figures 2, 3). No prior CT or MRI studies were available for comparison. She was discharged on D-penicillamine and phenytoin.

Discussion

Wilson's disease was first described as a syndrome by Kinnier Wilson in 1912 [2]. The peak age of presentation is between 8 and 16 years, though the condition can present at any age from 5 to 50 years or older [3]. ATP7B protein deficiency impairs biliary copper excretion, resulting in positive copper balance, hepatic copper accumulation and copper toxicity from oxidant damage. Defective copper incorporation into apoceruloplasmin leads to excess catabolism and low blood levels of ceruloplasmin. As the disease progresses, free copper levels increase causing deposition of copper in brain. The tests used to diagnose WD in increasing order of usefulness are: serum ceruloplasmin<Kayser-Fleischer (KF) rings<24-hour urine copper<liver Cu<haplotype analysis [1]. KF rings are present in >99% of patients with neurologic/psychiatric manifestation as in our case [1].

Neurological and neuropsychiatric signs are the presenting features in 40–50% of patients with Wilson's disease [4]. The neurological abnormalities can be classified as: (a) an akinetic-rigid syndrome similar to Parkinson's disease, (b) pseudosclerosis dominated by tremor, (c) ataxia, and (d) a dystonic syndrome which were not present in our case. Like few reported cases our patient had seizures as the only neurological sign [4].

Normally, the human brain has high copper levels in the putamen, globus pallidus and caudate nuclei, the substantia

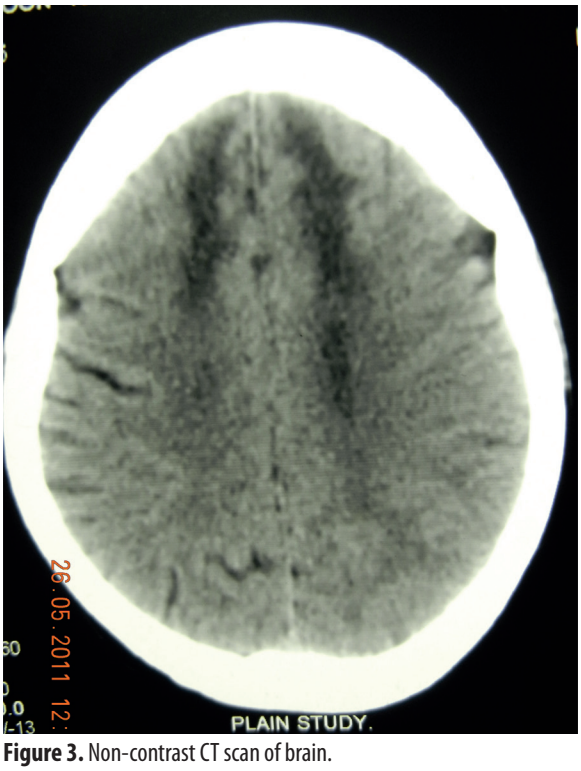

Figure 3. Non-contrast CT scan of brain.

nigra, locus caeruleus, and dentate nuclei. In Wilson's disease, abnormal deposition of copper is seen, especially in the putamen and globus pallidus. Spongy softening, cavitation, and a general reduction of neurons are a few histopathological changes that are seen in Wilson's disease involving the brain. Atrophic changes are seen in longstanding cases [5].

Structural brain MRI in patients with the disease has shown widespread lesions in the putamen, globus pallidus, caudate, thalamus, midbrain, pons, and

cerebellum as well as cortical atrophy and white matter changes. In general, these lesions show high-signal intensity on T2 weighted images and low-intensity on T1 scan. Although MRI changes are present in many Wilson's disease patients, even patients without neurological symptoms, these changes tend to be more severe and widespread in patients with neurological Wilson's disease [4].

Since there was no enhancement or mass effect in the CT, acute inflammatory or neoplastic disease could be ruled out. Generally, mild dilatations of ventricles indicate a neurodegenerative disorder (NDD). The possible NDD are Wilson's disease, Leigh disease, Huntington's disease and Hallervorden-Spatz disorder. Leigh's disease occurs in infancy or early childhood and CT shows bilaterally symmetrical lesions in thalamus, pons, brainstem, inferior olives and posterior column of spinal cord and uncommon in white matter and cerebral cortex, not seen in the index case. Huntington's disease occurs in fourth or fifth decade and presents with choreform movement disorder. CT shows caudate and putamen atrophy and cortical atrophy, especially in the frontal region. In Hallervorden-Spatz disorder CT shows hyper- or hypodensity in globus pallidus region in contrast to the presented case.

So, the CT changes were due to Wilson's disease which classically shows typical oblong bilateral hypodense lesions in thalamic and lentiform nuclei.

A CT scan study of our case showed the presence of bilateral symmetrical hypodensity at thalamo-capsular and frontal areas, not commonly found in WD. Also, minimal involvement of the lentiform nuclei was seen. No significant involvement of the cerebellar nuclei or superior or middle cerebellar peduncles was seen. Therefore, in a patient with similar CT scan findings following seizures, Wilson's disease should be kept as a differential.

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Experimental & Clinical
Hepatology

Received: 2011.09.19
Accepted: 2011.10.19
Published: 2011.12.15

Delayed reactivation of hepatitis B virus in a surface antigen negative patient treated for chronic lymphocytic leukaemia

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Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

Summary

Background: Reactivation of Hepatitis B Virus (HBV) is well documented in HBV surface antigen (HBsAg) positive patients undergoing immunosuppressive treatment. Guidelines exist for identifying and treating such at-risk patients. The aims of reporting this case are to highlight the importance of screening all patients undergoing immunosuppressive therapy and to discuss the options for the type and duration of treatment.

Case Report: We present a case of delayed *de novo* hepatitis B reactivation in a surface antigen negative patient with chronic lymphocytic leukaemia (CLL). The patient developed acute hepatitis B nine months after immunosuppressive treatment for CLL in 2009, having previously seroconverted after acute hepatitis B in 1994 (HBsAg-, HBcAB +). He was treated first with lamivudine in December 2009, adding tenofovir to treatment in January 2010 when further CLL treatment was planned and after showing signs of decompensated liver disease (abdominal ascites). We also include a review of the literature regarding delayed *de novo* hepatitis, factors to predict which patients might need treatment and also look at the use of nucleoside analogues and dual therapy in such patients.

Results: He remains on both lamivudine and tenofovir with normal liver function tests, no signs of liver disease, and undetectable HBsAg.

Conclusions: There are no other cases in the literature of a reverse seroconversion in a patient undergoing immunosuppressive chemotherapy for the second time.

Key words: **chronic leukemia • chronic viral hepatitis B • Tenofovir**

Abbreviations: **HBV** – hepatitis B virus; **CLL** – chronic lymphocytic leukaemia; **HBcAb** – hepatitis B core antibody; **LFTs** – liver function tests; **HBsAg** – hepatitis B surface antigen; **HBeAg** – hepatitis B envelope antigen; **SAAG** – serum albumin ascitic gradient; **AIHI** – autoimmune haemolytic anaemia; **ALT** – alanine transaminase; **HBc IgM** – hepatitis B core immunoglobulin M; **SMA** – smooth muscle antibody; **LKM** – Liver kidney microsomal antibody; **αFP** – alpha feto protein; **CMV** – cytomegalo virus; **ANA** – antinuclear antibody; **EBV** – Epstein Barr virus; **AMA** – anti mitochondrial antibody; **HCV** – hepatitis C virus; **ALP** – alkaline phosphatase; **PT** – prothrombin time

Full-text PDF: http://www.expclinhep.com/fulltxt.php?ICID=969168

Word count: 1574
Tables: 1
Figures: 2
References: 15

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Background

Reactivation of hepatitis B following treatment with chemotherapy is well documented. Current guidelines only recommend anti-viral prophylaxis for patients with hepatitis B surface antigen positivity. Recent cases of severe re-activation of hepatitis B have been documented in patients who were HBsAg-negative and had evidence of previous exposure to hepatitis B (core antibody positive). There is no clear guidance on how to manage these patients.

We present a case of severe hepatitis due to delayed reactivation of hepatitis B virus (HBV) in a 54 year old man nine months after completing a second course of chemotherapy with alemtuzamab and methylprednisolone for relapsing chronic lymphocytic leukaemia (CLL). He was HBsAg-negative and HB core antibody positive prior to treatment. He was treated with a combination of tenofovir and lamividine with good and rapid improvement.

Case Report

He first presented in 1994 aged 39 to a tertiary liver unit with fulminant liver failure secondary to acute HBV. He required intracranial pressure monitoring and was initially listed for urgent orthotopic liver transplant, but recovered spontaneously with supportive care. Liver biopsy at the time confirmed acute HBV. Subsequent viral serology confirmed surface antigen clearance with positive hepatitis B core antibody (HBcAb).

He was diagnosed with CLL Binet Stage B in 2005 aged 51 years. In late 2006 he underwent chemotherapy with six cycles of fludarabine and cyclophosphamide. His liver function remained normal throughout and he had no evidence of hepatitis. In July 2008 with massive lymph node enlargement and a white cell count of 375×10^9/l he received CamPred (alemtuzumab (Campath) and methyl prednisolone) therapy for clinically aggressive disease. Hepatitis B prophylaxis was not considered as he was HBsAg-negative. Liver function tests checked three monthly throughout treatment were normal.

In August 2009 (nine months after completing treatment with CamPred) he became jaundiced, anorectic and lethargic. Liver function tests (LFTs) and liver screen results are shown in Table 1, confirming acute hepatitis due to reactivation of hepatitis B virus. Abdominal ultrasound was normal apart from splenomegaly of 18 cm (likely multifactorial from his CLL and liver disease). By November 2009 after simple supportive care, his LFTs had significantly improved.

In December 2009 his CLL relapsed and further chemotherapy was planned. Due to his HBV status treatment with lamividine was planned. The chemotherapy was planned to start three months later. In late December of 2009 the patient developed ascites. His liver function tests did not show any significant deterioration. A diagnostic tap was performed which showed a high serum albumin-ascitic albumin gradient (SAAG), consistent with possible portal hypertension. A computed tomography scan reported extensive lymphadenopathy, large ascites, and right sided pleural effusion with no evidence of thrombosis in the portal vein or in the hepatic veins. A working diagnosis of decompensated liver disease due to Hepatitis B was made. He did not undergo liver biopsy as it was thought unlikely to affect his management at that stage. In view of the working diagnosis, tenofovir was added to his anti-viral treatment. His HBV DNA at this time was 1.3×10^6. His renal function and coagulation studies were completely normal. The patient showed good and rapid response to the treatment with significant improvement in liver function tests (Figure 1) and decrease in viral load (Figure 2). He had a transient deterioration during March 2010 due to severe pneumonia whilst on steroids for autoimmune haemolytic anaemia (AIHA) associated with CLL.

To date, he has not yet required any additional immunosuppressive treatment for his recurrent CLL other than five months of oral prednisolone for AIHA. He remains on tenofovir and lamivudine and has normal liver function tests with undetectable HBV DNA in October 2010.

His renal function has remained normal throughout, his ascites have now resolved, and he has not developed any signs of decompensated liver disease since.

Discussion

We present a case of delayed severe reactivation of hepatitis B which highlights a number of interesting and unique points for discussion.

- Our patient had been previously treated with chemotherapy without any adverse effects on his liver function,
- He had a reactivation "*De-novo* hepatitis" more than nine months post treatment,

Table 1. Liver screen and Liver function tests October 2009.

HBsAg +	CMV-neg	ANA-neg	IgA 0.8 (0.8-4.0)
HBcAb+	EBV-pos	AMA-neg	IgG 11.46 (6.0-16.0)
HBc IgM +		SMA 1:40	IgM 1.06 (0.5-1.9)
HBeAg +			LKM-neg
HBeAb equivocal			αFP 16.1
HCV-negative			
Bilirubin	153 umol/L	(<20)	
ALT	963 u/L	(5–30)	
ALP	331 u/L	(30–130)	
Albumin	35 g/L	(34–50)	
PT	17 s	(10–15)	

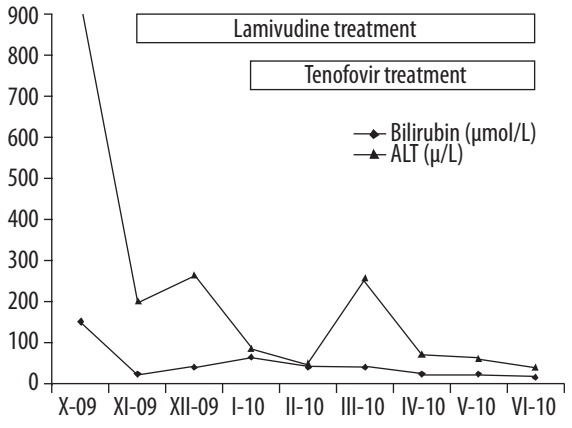

Figure 1. ALT and Bilirubin over time showing when HBV treatment started.

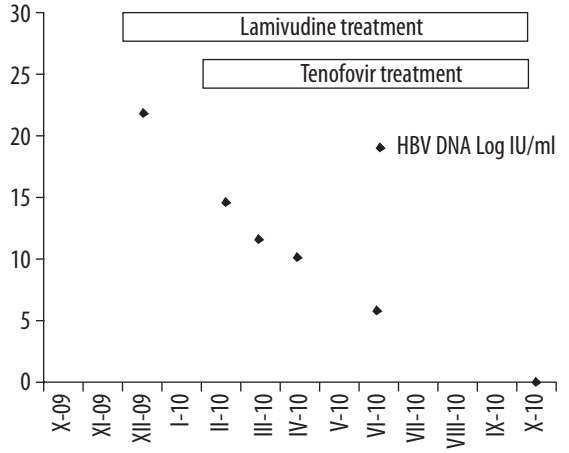

Figure 2. HBV DNA levels over time showing when HBV treatment started.

- Despite initial improvement of his hepatitis without specific treatment the patient developed hepatic decompensation with ascites,
- The patient had a good response to treatment with tenofovir and lamivudine with reversal of decompensation signs.

This case also raises some important questions about the indications for screening and prophylactic treatment for HBs antigen negative patients with previous exposure to hepatitis B (HBcAb positive). It also brings to discussion the role of the more potent nucleoside analogues in treating these patients, who often present with more aggressive disease.

Reactivation of hepatitis B is a syndrome characterised by the abrupt reappearance of HBV DNA in the serum of a patient with prior HBV infection. It can be spontaneous but is usually triggered by chemotherapy, immunosuppression or altered immune function. It is most frequently seen in HBsAg positive patients during or shortly after chemotherapy, in whom prophylaxis with lamivudine or other anti-virals is recommended in European and American guidelines [1,2]. A systematic review of the effect of lamivudine prophylaxis identified 14 studies with 750 patients. The relative risk for both HBV reactivation and HBV-related hepatitis ranged from 0.00 to 0.21 in the treatment group [3].

Reactivation in HBsAg negative patients is rare, occurring in 3–4% of patients after chemotherapy [4,5]. Referred to as "reverse seroconversion" or "*de novo* hepatitis B" it represents an aggressive form of HBV reactivation with a high associated morbidity and mortality.

More recently reactivation in HBsAg negative patients has been reported with the use of rituximab immunotherapy. Rituximab (a monoclonal antibody against CD20, a major cell surface marker on B cells) is associated with high levels of HBV reactivation and high mortality [6–12]. In five of these case reports the patients were HBsAg negative as in our case, and occurred late after several cycles of chemotherapy with rituximab. One of these cases reports an HBsAg negative patient successfully treated with entacavir for his reactivation [5]. The authors suggest that patients being considered for chemotherapy be screened with HBsAg, anti HBs and anti HBc. They also recommend continuation of prophylaxis for at least 12 months after cessation of chemotherapy. Our case clearly supports these recommendations, even for those patients not receiving rituximab, but on other highly immunosuppressive treatment such as alemtuzamab and corticosteroids.

Predicting which patients will reactivate is difficult. Studies have shown HBeAg positive and HBV DNA positive patients and those receiving chemotherapy that includes corticosteroids are most at risk [13,14]. A study in 2000 [15] also identified younger male patients at higher risk. The aggressiveness and duration of the chemotherapy or immune suppression is also a key factor, as demonstrated by the high incidence of reactivation with rituximab.

There are no other cases in the literature of a reverse seroconversion, in a patient undergoing immunosuppressive chemotherapy for the second time.

It is unclear why our patient did not reactivate at the time of his first chemotherapy in 2006 yet did after CamPred in 2008/9. One possibility is that CamPred targets CD52 and both B and T cell mediated immune responses. It is even more potent than fludarabine and cyclophosphamide, specifically being more toxic to T cells which may increase the risk of HBV reactivation. CamPred is not as widely used as rituximab and hence problems associated with reactivation are reported less often in the literature.

Another unexplained feature in this case is the nine month delay between his second course of chemotherapy and reactivation. This has not previously been reported. The appropriate length of antiviral treatment beyond completion of chemotherapy is not consistent from the reports in the literature, although the American Association for the Study of Liver Diseases (AASLD) guidelines recommend "up to 12 months" [2]. Current European (EASL) and AASLD guidelines recommend that HBsAg negative patients are monitored with ALT and do not routinely need prophylactic drug treatment [1,2]. This case report adds to the evidence that prophylaxis ought to be considered in *all* patients with

a past history of HBV regardless of the HBsAg status at the time chemo/immunotherapy is commenced. This may be particularly important if more toxic/T cell targeted therapy is planned (e.g.CamPred). The case also highlights the importance of continued treatment with anti-viral drugs once therapy is complete, and regular monitoring of HBV DNA. The duration of monitoring and HBV treatment after reactivation is currently unclear, although our report would support the case for at least 12 months. The issue of when it is safe to recommence chemo/immunotherapy remains uncertain, but clearly waiting until HBV DNA is undetectable is the only measurable guide to this.

Treatment with both lamivudine and tenofovir in HBV reactivation is not reported. Our patient had signs of decompensated disease and with additional immunotherapy planned the addition of a second agent was appropriate. His HBV DNA levels and ALT have both responded and he has had no adverse reactions to dual therapy. We plan to continue both treatments for 12 months and then if further CLL therapy is required restart lamivudine in the first instance.

Conclusions

This is the first reported case of delayed de novo hepatitis B reactivation in a surface antigen negative patient with CLL undergoing chemotherapy for the second time. It highlights the importance of considering prophylactic antiviral therapy in all patients with a past history of HBV regardless of the HBsAg status at the time chemo/immunotherapy is commenced. The ideal duration of chemoprophylaxis in such patients remains unclear.

Acknowledgements and Disclosures

None.

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Experimental & Clinical Hepatology

Received: 2011.07.24
Accepted: 2011.09.03
Published: 2011.12.15

Hepatocellular carcinoma (HCC) as a consequence of chronic hepatitis. Epidemiological and clinical aspects

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Source of support: National Center for Investigation and Development, Ministry of Science and Higher Education, grant Nr 12710

Summary

Hepatocellular carcinoma (HCC) represents a major challenge in contemporary medicine, being one of the most common malignancy worldwide and a leading oncological cause of mortality. HCC incidence is increasing mainly due to the prevalence of chronic viral hepatitis leading to liver cirrhosis. HCC develops in a cirrhotic liver in a majority of cases, and the pre-neoplastic condition is the strongest predisposing factor. Emerging data indicate that mortality from non-HCC complications of cirrhosis is decreasing or stable, whereas mortality rate of HCC is rising, therefore becoming the main cause of liver-related death. Because the at-risk population can easily be identified, mass screening conducted as early as possible seems to be justified, as surgery interventions at an early stage can substantially increase survival chances.

The present review focuses on two main aspects of the HCC problem according to the updated HCC recommendations: the epidemiological aspect with HCC prevalence and specification of characteristic HCC risk groups of patients, and the clinical aspect - with diagnostic difficulties and therapeutic dilemmas.

Key words: **hepatocellular carcinoma • liver cirrhosis • chronic hepatitis**

Full-text PDF: http://www.expclinhep.com/fulltxt.php?ICID=969169

Word count: 4238
Tables: 3
Figures: 1
References: 62

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Background

Hepatocellular carcinoma (HCC) represents one of the most common malignancies and a leading cause of cancer-related deaths worldwide [1,2]. Prevalence of HCC is geographically differentiated, with the highest incidence rates in Subsaharic Africa and South-East Asia [1,2]. The countries with relatively small incidence rates include Poland, however HCC is responsible there for numerous deaths every year, and statistically observed trends suggest that mortality rates will continue increasing mainly due to the prevalence of chronic hepatitis C and B [3,4]. HCC usually develops on the background of liver cirrhosis, and this pre-neoplastic condition is the strongest predisposing factor. Emerging data indicate that mortality from non-HCC complications of cirrhosis is decreasing or stable, whereas mortality rate of HCC is rising, therefore becoming the main cause of liver-related deaths among patients with compensated liver cirrhosis. Potentially curative treatment is only limited to cases with a relatively early stage of HCC liver changes. Thus, screening based on ultrasound imaging is recommended every 6 months in all patients of HCC risk groups. Detection of HCC at an early stage may reduce mortality significantly. Because the at-risk population can easily be identified, mass screening conducted as early as possible seems to be justified, as surgery interventions at an early stage can substantially increase survival chances [5]. The review briefly presents 2 main aspects of HCC problem according to the updated HCC recommendations [6–8]: the epidemiological aspect with HCC prevalence and specification of characteristic HCC risk groups of patients, and the clinical aspect – with diagnostic difficulties and therapeutic dilemmas.

The Range of HCC Problem – Epidemiological Aspects

Chronic viral hepatitis as the HCC risk factors

Approximately 5% of the world population, that is 350 million people, are chronically infected with HBV [9]. Approximately 3% of the world population, that is 170 million people, are chronically infected with HCV [10]. A much worse prognosis of HBV/HCV co-infections concerns 30 million patients, resulting in a total sum of nearly half a billion infected, of whom one in five on average, after a period of 20 years of mostly clinically silent infection, will manifest difficult-to-control symptoms of liver cirrhosis with hepatocellular carcinoma (HCC) [3] which has proved to be the main cause of liver-related deaths.

In Poland, similarly to most European countries, the USA and Japan, long aspect ratio on the percentage of HBV and HCV chronic carriers is reversed. About 350 thousand Poles are chronically HBV-infected, whereas approximately 750 thousand are HCV-infected. Given the occurrence of approximately 10% of patients with HBV/HCV co-infections, the number of chronically infected with viruses which are originally hepatotropic [3] reaches almost one million in Poland. The improvement of the epidemiological situation of hepatitis B is mostly due to the introduction of the HBV vaccination in the middle of the 1990's, thus contributing to a significant decline of approximately 60% in incidence of HCC. The availability of safe and effective vaccines allowed wide immunization programmes to be conducted, which led to a reduction of the number of diseases caused by HBV, with clear benefits in terms of prevention of cirrhosis and HCC. The seemingly simple hepatitis B vaccine has proved to be the vaccine for liver cancer [11,12] (Table 1).

The separation proposed by experts (AASLD Practice Guideline) [6–8] of Asian and African populations as high-risk groups particularly vulnerable to the occurrence of HCC results from the highest incidence rates for HCC in Subsaharic Africa and South-East Asia (up to 120/100 000). It clearly correlates with the highest number of the HBV-infected, reaching up to 30% of the population in these regions [13].

Perinatal transmission and transmission during early childhood are responsible for the high rate of chronic infection in Asia and Africa, therefore the presence of HCC liver changes at relatively young men, without earlier diagnosed cirrhosis is reported there more frequently [13]. This situation is due to direct prooncogenic activity of the HBV virus which, in contrast to the HCV, induces neoplasmal transformation by integrating into the DNA of infected hepatocytes, i.a. through the expression of proteins HBX and LHBs [14]. Tests carried out on the population of Taiwan have shown that in the absence of HBV infection HCC incidence rates are low and amount to about 5/100 000 per year, and in the presence of HBV infection they range to 1000/100 000 yearly [15].

Chronic hepatitis B is responsible for at least 75% of HCC. HBV-related end-stage liver disease and HCC cause over 1 million deaths yearly and currently represent 5–10% of cases of liver transplantation [16,17]. It has been confirmed that the risk of developing HCC increases in proportion to the increase in HBV viraemia [12], which, however, does not diminish the new problem of minireplications in the occult hepatitis B. Studies show that the mechanisms of

Table 1. HCC specific risk groups [6–8].

Hepatitis B carriers	Non-hepatitis B carriers
Cirrhotic hepatitis B carriers (incidence of HCC: 3–8%/year)	Hepatitis C cirrhosis (incidence of HCC: 3–5%/year)
Family history of HCC (Incidence higher than without history)	Stage 4 primary biliary cirrhosis (incidence of HCC: 3–5%/year)
Asian males >40 years (incidence of HCC: 0.4–0.6%/year)	Genetic hemochromatosis and cirrhosis (incidence >1.5%/year)
Asian females >50 years (incidence of HCC: 0.3–0.6%/year)	Alpha1-antitrypsin deficiency and cirrhosis (incidence >1.5%/year)
African/North American Blacs (HCC occurs at a younger age)	Other cirrhosis (incidence unknown)

liver carcinogenesis in the course of latent infection are the same as in non-latent infection and as the incidence of occult hepatitis B in patients with diagnosed HCC is 22–87% [18,19]. Most diagnoses of occult HBV infection indicate patients chronically co-infected with the HCV, that is over 30% of cases, mainly due to common hematogenous transmission route of infection. The situation of undetectable HBs antigen in the presence of HBV DNA in hepatocytes, (rarely in the serum), relatively frequently occuring in this group of patients, is the result of proven HCV virus inhibition on the HBV replication [14]. Therefore, it may be assumed that HBV/HCV co-infections accelerate progression of liver disease, similarly to the frequently neglected HDV superinfection, which increases the frequency of development of chronic hepatitis B with further clinical implications from 5% to 80%.

Increasing prevalence of HCV infections, which may be observed in developed countries, is associated primarily with the development of diagnostic methods (especially molecular biology techniques) and their more popular use. As a consequence, there has been observed an increase in registration of undetected hepatitis acquired in the past [3]. Identifications of asymptomatic acute hepatitis C are relatively rare. However, new HCV infections still occur – the estimate specifies about 4 million yearly worldwide. Global prevalence of chronic hepatitis C, lower than hepatitis B and ranging from 0,1 to 5% in different countries [20], proves to be the most frequent cause of HCC in economically developed regions. In industralized countries, HCV accounts for 20% of cases of acute hepatitis, 70% of cases of chronic hepatitis, 40% of cases of end-stage cirrhosis, 30% of liver transplants and 60% of cases of HCC [21]. Chronic HCV infections represent a precancerous state accompanied by increased DNA synthesis [22,23]. Data show that approximately 10–20% of liver cirrhotic patients may develop HCC within 5 years [24,25]. Recent cohort studies have indicated that HCC is the most frequent cause of death in patients infected with HCV, and epidemiological trends suggest that the mortality rate is rising [26]. The annual rate of HCC developing among patients with HCV-associated cirrhosis is estimated to be 1.5–2.5% [27]. For unspecified reasons, Japan and Taiwan fall outside this range, with rate of more than 7%. Japan, however, has observed a decline [28]. The annual death rate among patients with HCC also depends on the effectiveness of HCC surveillance strategies and on the access to potentially curative treatment which in industrialized countries is estimated to be around 80%, whereas in developing countries is around 90%. In Japan the annual mortality rate is significantly lower – <10% [29,30].

HIV-infected patients have increased incidence of HBV and HCV infection because of the same transmission routes, hence being at a greater risk of liver damage progression and HCC. The use of highly active antiretroviral treatment has prolonged lives of HIV-patients, whereas HCC has become the leading non-AIDS cause of death. The main characteristics of HCC in HIV-infected patients and their survival have been poorly described so far. There has been no clear evidence yet as to what impact the HIV itself has on the risk of HCC infection, or what the accelerated effect of HBV and/or HCV co-infections is on the HCC development. Clifford et al. [31] in 2008 performed a case-control study of HCC nested within the Swiss HIV Cohort Study to determine the effect of immune suppresion on the development of HCC for HIV-infected patients. Only 26 patients with HCC were identified: 10 HCC+HIV patients had HBV co-infection, whereas 11 had HCV co-infection, and 5 had both co-infections. Investigators found that CD4 cell count taken within a year of diagnosis was associated with risk of HCC: each 100cells/mm^3 decrease was associated with a 33% increase in HCC risk. Salmon-Ceron et al. [32] in France anlyzed retrospectively the effect of HIV co-infection on the HCC development. The French national Mortalite 2005 study listed 1042 deaths, 44% of which with reported co-infections with hepatitis virus and 138 with liver-related deaths caused by decompensated liver cirrhosis in 66% and by HCC in 25%. HCC increased among liver-related deaths from 16/110 (15%) in 2000 to 35/138 (25%) in 2005, despite improved control of HIV infection (average CD4 cell counts 157 in 2000 *vs.* 231 in 2005).

Steatohepatitis as the HCC risk factor

Very frequently asymptomatic and undiagnosed chronic viral hepatitis infections overlap with increasingly common issues of modern civilization, which seemingly accelerate progression of an already developing disease. While obesity is considered to be the real epidemic of XXI century, it is estimated that over 20% of the population, mainly in the USA and Western countries, presents the problem and develops clinical consequences as a metabolic syndrome with diabetes, dyslipidemia and/or arterial hypertension. Hepatic manifestation of the disorder is non-alcoholic steatohepatitis (NASH). NASH seems to be an important cofactor affecting progression towards fibrosis. It is known that insulin resistance induces liver fatty degeneration through increasing hepatic lipolysis in the mechanism of activation of microsomal lipooxygenase cytochrome P-450 2E1 with secondary release of free radicals and local oxidative stress causing inflammation and fibrosis [33,34]. One intervention study suggests that rational reduction in weight leads to reduction in fibrosis progression [35].

Similar liver steatosis is also considered the most common form of alcoholic hepatopathy accelerating progression of chronic hepatitis C into liver cirrhosis with an average of 20 years for a period of about 10 years, which occurs in 70% of cases infected with HCV. It is confirmed that the intake of more than 50 g alcohol/day accelerates progression to cirrhosis with a relative risk of about three [36,37].

Other important HCC risk factors

Numerous other rare disorders or clinical conditions cause predisposition to liver carcinogenesis through progression of chronic hepatitis, the most important being hemochromatosis, autoimmune hepatitis, primary biliary cirrhosis (PBC) and primary scleroticans cholangitis (PSC), alpha-1 antytrypsin deficiency, nutritional deficiencies (kwashiorkor), tyrosinemia, skin late and acute intermittent porphyrias, some glikogenosis, Budd-Chiari syndrome and Wilson's disease [3,38]. It has been reported that oral anabolic steroids and hormonal contraception may also contribute to the hepatocarcinogenesis. There is also some evidence suggesting that daily cigarette [39] and cannabis [40] smoking may influence the development of HCC. Among carcinogens there are hepatotoxins such as nitrosamines, carbon

Table 2. HCC risk factors [41].

	Europe	North America	Asia & Africa	Japan
HCV	60–70%	50–60%	20%	70%
HBV	10–15%	20%	70%	10–20%
ALC	20%	20%	10%	10%
Others	10%	10%	0%	0%

tetrachloride, p-dimethylamino-benzene, vinyl chloride, or mycotoxins of Aspergillus flavus resulting from improper storage of cereal grains, maize and nuts mainly in Asia and Africa. Still less is known of the mechanisms of interaction between aflatoxin B1 and endemic HBV infection which cause the world's highest incidence rates for HCC in the two continents [3,38]. Chronic hepatitis is known to induce liver fibrosis and to change hepatic cytoangio-architecture, contributing to the loss of control over cell division and, as a consequence, to multiplication of genetic errors and mutations, which causes neoplasmal transformation (Table 2).

The Weight of HCC Problem – Clinical Aspects

Diagnostic difficulties

Blood from over than 30% of the cardiac output flows through the liver. Therefore liver is the place where hematogenous metastases have frequently been found, as confirmed in more than 30% of patients with malignant tumors by post-mortem pathological examinations. Hepatic metastases occur 30 times more frequently than hepatocellular carcinoma, hepatologists, however, more frequently encounter focal liver changes specific for HCC.

The majority of HCC cases, constituting more than 85%, present underlying liver cirrhosis which masks manifestations of the neoplasmal progression. The clinical course of HCC is untypical and often asymptomatic. Therefore in cirrhotic patients any of the general disorders should be considered alarming, among them particularly those not yet occuring, such as abdominal pain, fever, progressive weight loss and cachexia, as well as increasing hepatic decompensation with jaundice and ascites. Symptoms of paraneoplastic syndrome, such as policytemia, hypercalcemia or hipoglycaemia, have also been observed [41].

Most frequently, suspicious focal liver changes are detected accidentally while monitoring the patient's condition during the popular abdominal ultrasound examination. For this reason diagnosis based on ultrasound imaging conducted every 6 months is recommended for all patients of HCC risk groups (Table 1) [6–8], with the surveillance interval proposed on the basis of tumour doubling times. Ultrasound (US) used as a screening test has been reported to have a sensitivity of 65–80% and a specificity of 48–94% [42,43]. Diagnostic success of US for HCC surveillance strategy depends on numerous factors, but mostly on the size and character of the focal liver changes, as well as the experience of the sonographist and the technical quality of the ultrasound equipment. The value of US surveillance performed in a primary care setting by operators who do not have specific skills is questionable. Ultrasound detection of HCC, especially on a cirrhotic background, presents a challenging issue even in expert hands. Liver cirrhosis is characterized by fibrous septa and regenerative nodules. These features produce a coarse pattern on ultrasound that may impair identification of small tumours which are usually homogenic and hypoechogenic. Because tumours increase forming focal necrosis and micro-bleeding, they become more and more heterogenic and hyperechogenic, as well as more visible and detectable for imaging techniques. Unfortunately, this feature together with arterial vascularity is typical for higher malignancy and worse prognosis. It is the neovascularity that allows HCC to be diagnosed and is the key to the imaging of cirrhotic patients. Doppler's functions or contrast-enhanced US can be used for clearer differentiation of the advanced changes, leading to a better visualisation of the relation between organic neoplasma and vascular structures [42,43]. Because ultrasound examination is subjective and non-repetitive, all focal liver changes suspected on US should be verified with contrast-enhanced computer tomography (CT) and/or magnetic resonance imaging (MRI), as the use of these methods tends to lead to more accurate diagnosis of HCC, with sensitivity up to 89% and specificity reaching 99% [43]. Unfortunately the diagnosis does not seem very precise in the rarer cases of the smallest lesions of less than 1 cm in diameter – reaching as little as 34% [44].

Numerous epidemiological data have confirmed that HCC are 50% of lesions smaller than 1 cm in diameter on ultrasound. According to the novel recommendations [7,8], those changes should be observed, or, more precisely, screened by US with 3 month intervals. If no growth has been observed over a period of up to 2 years, routine surveillance with intervals of 6 months can be undertaken again. Lesions of over 1 cm in diameter detected on US screening of a cirrhotic liver should be verified with 4-phase multidetector CT scan or dynamic contrast enhanced MRI. HCC is diagnosed if the features of the lesions are typical: with hypervascularisation in the arterial phase and washout in the portal venous or delayed phase. Another contrast-enhanced radiological study or a biopsy of the lesion should be carried out if the findings are not characteristic or the vascular profile is not typical [7,8].

The distinction between the early HCC changes and dysplastic nodules among cirrhotic patients tends to be a major challenge even in expert hands. It frequently proves very difficult to characterize solely by available radiological techniques and biopsies [45]. Expert pathology diagnosis may be supported by staining for glypican 3, heat shock protein 70, and glutamine synthetase, due to their high

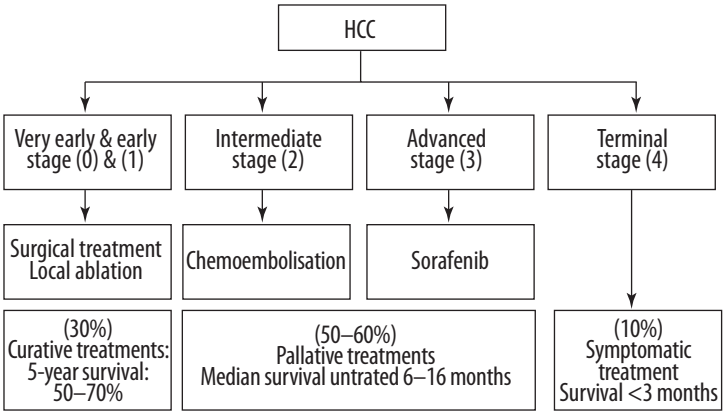

Figure 1. The Barcelona Clinic Liver Cancer (BCLC) staging system – the recommended staging system for HCC [5–8,49,50].

Table 3. Child-Pough classicifaction of severity of liver disease [52].

	1	2	3
Albumin [g/dL]	>3.5	2.8–3.5	<2.8
Bilirrubin [mg/dL]	1.0–2.0	2.0–3.0	>3.0
Prothrombin time [sec]	<4.0	4.0–6.0	>6.0
Encephalopathy	None	Grade I–II	Grade III–IV
Ascites	Absent	Slight	Moderate
Pugh score	5–6	7–9	10–15
Child score	Child A	Child B	Child C

accuracy for HCC detection [5–8]. Recent studies have revealed poor sensitivity and specificity of alpha-fetoprotein (AFP) determination for effective surveillance and diagnosis [8]. Therefore, surveillance should be based on US, and diagnosis needs contrast-enhanced imaging verification and/or biopsies without AFP testing. Numerous serum biomarkers such as des-gamma carboxyprothromin (DCP), alpha-1-fucosidase (AFU), glypican-3 (GPC-3) present significant diagnostic limitations, and therefore lack precision for the early diagnosis of HCC. The accuracy in differentiating HCC from nonmalignant hepathopathy could be improved by simultaneous determination of these markers in various combinations [46,47]. The most promising as a novel tool to improve diagnostic and prognostic prediction seems to be gene-expression profiling as it clearly introduces a new hope in supporting the visual techniques for early HCC diagnosis [48]. However, more studies are needed to demonstrate its superiority.

Therapeutic dilemmas

The therapeutic options are determined by the stage of HCC, that is by its size and location, the state of the liver not occupied by the neoplasmal process, and the general psycho-physical condition of the patient. Most major trials of HCC therapy have chosen the Barcelona Clinic Liver Cancer (BCLC) staging system, making it the reference staging system for HCC management [49,50]. It identifies patients with HCC who may benefit from curative treatments, patients at intermediate or advanced disease stage who may benefit from palliative treatments, as well as patients at terminal stage with a very poor life expectancy. Early stage disease (1) includes patients with preserved liver function (Child-Pugh A and B) with solitary HCC or up to 3 nodules less than 3 cm in size. Very early HCC (0) is understood as a single lesion less than 2 cm in size. The intermediate stage (2) consists of Child-Pugh A and B patients with large/multifocal HCC who do not have cancer related symptoms and do not have macrovascular invasion or extrahepatic spread. Patients with cancer symptoms and/or with vascular invasion or extrahepatic spread represent the advanced stage (3). Finally, terminal stage (4) are patients with extensive tumor involvement leading to severe deterioration of their physical capacity and/or major impairment of liver function (Child-Pugh C) [6–8,49,50] (Figure 1).

The treatment of choice for patients with the optimal profile, as defined the BCLC staging system: with localized HCC and good liver function has proved to be radical surgical resection [3,51]. However, because of too late detection of HCC mainly among patients with underlying advanced liver cirrhosis, resection can be performed only in every fifth patient with only a 50% chance for therapeutic success [51]. Selection of candidates for liver resection is crucial for the risk of severe postoperative complications caused by postoperative liver failure to be diminished. For this reason excluded are: cirrhotic patients in group C by Child score (in group B possible is only limited resection), and all those who

present: ascites, significant portal hypertension, thrombocytopenia (<100 000/mm^3) or elevated activity of transaminases (>2 fold) [3,51]. The Indocyanine Green retention test is also usually used in Asian countries to preoperatively asses the liver function (Table 3).

Due to progress in surgical treatment and proper patient selection in experienced centers, the treatment related mortality is less than 3% in these patients. Thus, 5-year survival rates of 70% or more are achievable, if the risk of tumour recurrence is low. Numerous data indicate that HCC recurrence is predisposed by the stage of cirrhosis (A<B<C by Child score) and invasiveness of cancer (multiple tumors; diameter of tumor >5 cm; small tumor histological differentiation; no bag and the presence of satellite nodules; vascular invasion). There is estimated HCC recurrence applicable to up to 40% of patients after 1-year of resection, 60% – after 2 years, 80% – after 5 years [3,51,53]. The cause of oncogenesis is the cirrhosis of the entire organ, so the best results of resective treatment are obtained for a relatively small proportion of patients with non-cirrhotic livers – only 5% of the cases in the Western world, and approximately 40% in Asian countries. In this group 80% of patients have made a radical treatment, while 5-year survival applies to 67% of the operated on, and HCC recurrence is observed only in 16% of the treated [6].

In a cohort study of radiofrequency ablation, complete ablation of lesions smaller than 2 cm proved possible in more than 90% of cases, with a local recurrence rate less than 1% [54]. More studies are needed before positioning ablation as the firstline strategy for very early HCC. However, according to recommendations for HCC management local ablation with radiofrequency or alcohol injection is considered a safe and effective therapy for patients who cannot undergo resection, as well as a bridge to transplantation [7,8].

For patients with multifocal HCC and/or decompensated liver cirrhosis excluded from radical resection, the best therapeutical option proves to be liver transplantation if no general contraindications are present. To minimize the risk of tumor recurrence after transplantation during chronic immunosuppression, the crucial factor is the appropriate selection of patients. Many centers accept for liver transplantation only patients who have fulfilled the Milan criteria: one tumor <5cm or max. 3 nodules with each nodule <3 cm, without vascular infiltration and local and distant metastases. In patients fulfilling these criteria, the 5-year survival rate is higher than 70%, as confirmed in numerous studies with larger numbers of patients [3,5,55,56].

For patients with confirmed unresectable HCC who do not meet the above criteria for liver transplantation, a series of palliative treatments is suggested with tumor embolisation and chemotherapy, which, although does not significantly prolong their lives, frequently, however, significantly improves their quality of life comfort [3,5–8,55]. Recently special attention have been directed into downstaging therapies. In the HCC treatment, therapies such as trans-arterial chemoembolisation (TACE), trans-arterial radio-embolisation (TARE), percutaneous ethanol injection (PEI) and radio-frequency ablation (RFA) can decrease the size with overall viability of the tumours, thus potentially increasing the proportion of patients qualifying for resection and transplantation [57].

Oncological methods, such as external radiotherapy and systemic chemotherapy, have not yet proved successful in the treatment of patients with HCC, allowing for up to 20% therapeutic response with common presence of side effects which significantly degrade the quality of life – e.g. PIAF. Currently, selective chemoembolization of artery, which afferents blood directly into the tumor proves to be much more effective. Transarterial chemoembolisation (TACE) is a treatment option for patients with non-resectable HCC, disqualified from or awaiting liver transplantation. It seems most effective when doxorubicin, mitomycin C, epirubicin or cisplatin [5,58] is used.

In recent years, the advancement of knowledge on molecular aspects of HCC pathogenesis has resulted in a new trend of oncological treatment. Targeted molecular therapies have raised a great deal of hope, with numerous ongoing clinical trials assessing their real performance [5,59]. Probably the best known among them is sorafenib: a multikinase inhibitor, whose antiproliferative, proapoptotic and antiangiogenic effects were well documented in the preclinical development of the drug. A large, prospective randomized, placebo-controlled trial of sorafenib in the advanced stage of HCC – SHARP (Sorafenib HCC Assessment Randomized Protocol) – with preserved liver function (non-cirrhotics or Child-Pugh A patients), has indicated that sorafenib has been able to improve the overall survival from 7,9 months to 10,7 months. The trial has lead to the worldwide registration of sorafenib for the treatment of HCC at advanced stage [5,59].

Conclusions

HCC can arise in both non-cirrhotic and cirrhotic livers. In the Orient, however, where hepatitis B or toxins are found to be the most common underlying causes, HCC commonly arises in the absence of cirrhosis. In the West, on the other hand, where hepatitis C and alcohol are the most common underlying liver diseases, HCC arises mostly in the setting of cirrhosis. Prognosis is predictably worse in patients with underlying cirrhosis and is determined not only by factor related to the tumour, but also by factors related to cirrhosis. HCC is responsible for significant morbidity and mortality in cirrhosis, it frequently leads to decompensation of cirrhosis, and is one of the leading causes of death. Potentially curative treatment for HCC patients includes resection and transplantation. Resection can be performed in patients with good liver function, and localised HCCs, while transplantation is favoured in selected patients with decreased liver function and/or multiple nodules. Over the years, the place of the therapies has been well defined, but they can only be attempted in 10–20% of patients with HCC, as in the majority of patients the disease will be too advanced [60,61]. A broader use of local HCC treatments has the rescue potential to shrink the tumour and to allow a curative option. However, because of serious limitations of curative tratment available, the most important challenge seems to be the earliest possible, particularly sensitive detection of resectable focal liver changes. In the nineties in Europe, lesions less than 2 cm in diameter represented <5% of the cases, whereas nowadays they represent up to 30% of cases in Japan [5]. Substantially more effective surveillance strategies in Japan have led to earlier HCC detection and earlier qualification for radical surgery, with very good postoperative survival rates and without perioperative mortality

[62]. With the use of such data European and American experts have defined trends and expected aims of surveillance policies in Western countries for 1980–2020. Access to potentially curative treatments has been classified into three periods: 5–10% of cases – until 1990; 30–40% of cases between 1990–2010; and 40–60% of cases in 2010–2020 [5]. As detection of HCC at an early stage may reduce mortality significantly, mass screening may be justified because the at-risk population can easily be identified, whereas surgery interventions at an early stage can significantly increase survival chances.

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