Experimental & Clinical Hepatology

Received: 2012.08.31
Accepted: 2012.09.14
Published: 2012.09.18

A rare presentation of multiple large primary extramedullary hepatic plasmacytoma

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

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Source of support: Departmental sources

Summary

Background: Extramedullary hepatic plasmacytomas are uncommon. Although a few case reports of extramedullary hepatic plasmacytoma have been published, we could not find any report on large and multiple primary extramedullary hepatic plasmacytomas in the literature.

Case Report: A 54-year-old woman who presented with abdominal pain, a 2-month history of weight loss (3 kg) and enlargement of the liver, is described. Histological examination of liver biopsy material showed sheets of monomorphic plasmacytoid cells. Immunohistochemistry confirmed monoclonal IgA and Kappa light chain expression. Bone marrow revealed no abnormalities. The patient was treated with chemotherapy. One year after diagnosis, the liver mass disappeared.

Conclusions: Extramedullary appearances, still not that common, should be considered and multiple myeloma should be reserved as a widespread differential diagnosis of SOL of the liver. Biopsy was necessary to reach the final diagnosis. It is important to differentiate plasmacytoma from other hepatic metastases.

Key words: liver • plasmacytoma • imaging • cytology

Full-text PDF: http://www.expclinhep.com/fulltxt.php?ICID=883448

Word count: 973
Tables: –
Figures: 4
References: 15

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Background

Multiple myeloma encompasses primarily the bone marrow and causes skeletal destruction, renal failure, anemia, and hypercalcemia [1]. Extramedullary plasmacytoma has been defined as an autonomous proliferation of monoclonal plasma cells with varying degrees of differentation, producing a circumscribed mass or diffuse infiltration [1]. The most common sites are the head, neck, lymph nodes, lungs and skin [2]. Even though plasma cell infiltration of the liver is detected in around 40% of patients with multiple myeloma at post-mortem [3], it is not often distinguished in living patients. Only a few cases of primary hepatic extramedually plasmacytoma have been reported. We report on an isolated multiple large hepatic extramedually plasmacytoma without any evidence of myeloma elsewhere.

Case Report

A 54-year-old woman presented with a 2-week history of abdominal pain, and a 2-month history of weight loss of 3 kg. No history of vomiting or fever. She had been known to be diabetic and hypertensive for the past one year. On physical examination she was conscious. There was no significant pallor. On abdominal examination, there was moderate enlargement of the liver. No tenderness. Bowel sounds were normal. Rectal examination was normal. Abdominal ultrasound showed heteroechoic lesions measuring 12.7±6.1, 4.7±5.7 and 4.5±5.5 cm, visible in the right lobe of the liver. CECT of abdomen and pelvis showed enlarged liver, large hypodense mass lesions in the right hepatic lobe involving 5,6,7,8. The following hypodense lesions were found: of 5.7cm in segment 2, 4.8 cm in segment 4B, 2.5 cm in segment 5, and 1.4 cm in segment 7 (Figure 1). Gallbladder, common bile duct and pancreas were normal. Portal and hepatic veins appeared normal. Skeletal radiography revealed no abnormalities. Haematological indexes, liver function test, and renal function tests were within normal limits. The patient was negative for hepatitis B virus surface antigen and hepatitis C virus. Alpha fetoprotein was not increased. Serum protein electrophoresis showed increased gamma globulins, while serum immuno-electrophoresis revealed an IgA Kappa monoclonal gammopathy.

Serum beta-2microglobulin was increased to 0.45 mg/dL (normal value: 0.11–0.24 mg/dL). A needle biopsy of the liver and bone marrow trephine biopsies were performed. The biopsy specimens were fixed in 4% formaldehyde, embedded in paraffin wax and stained with haematoxylin and eosin and Congo red. Histological examination of liver biopsy specimens showed sheets of monomorphic plasmacytoid cells with eccentrically placed nuclei, eosinophilic cytoplasm, and perinuclear clearing (Figure 2). The background was rich in pinkish amorphous hyaline material which was negative for amyloid on Congo red stain. The immunohistochemical analysis strongly suggested liver plasmacytoma of IgA kappa type with strong membranous positivity of CD138 (Figures 3, 4). The bone marrow biopsy specimens showed hypercellular marrow, normal otherwise. Serum calcium, serum and urine protein immunoelectrophoresis were normal. Therefore, a diagnosis of extramedullary liver plasmacytoma was made and the patient was treated with Inj Bortezomib and Inj Dexamethasone. One year after the diagnosis, the liver mass disappeared. There was no

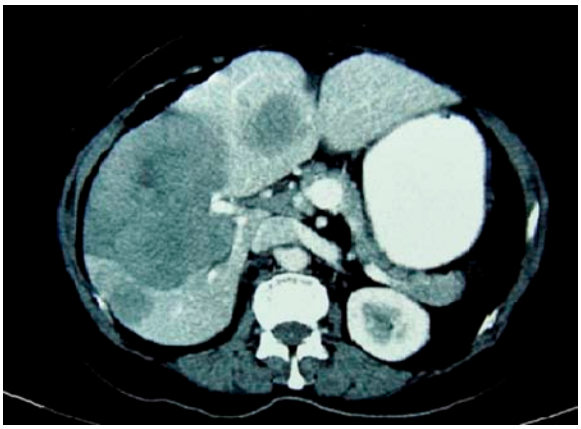

Figure 1. CECT of the abdomen and pelvis showing enlarged liver, multiple large hypodense mass lesions in the right lobe.

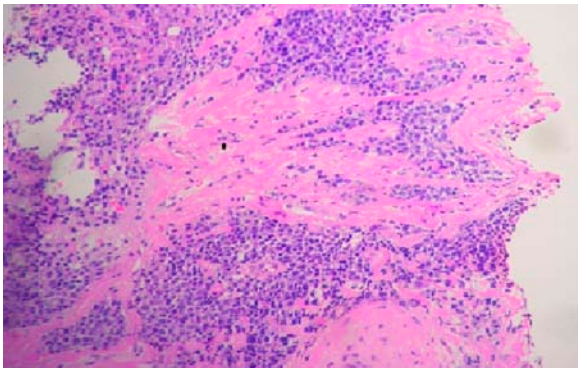

Figure 2. Histological examination showing sheets of monomorphic plasmacytoid cells with eccentrically placed nuclei, eosinophilic cytoplasm, and perinuclear clearing.

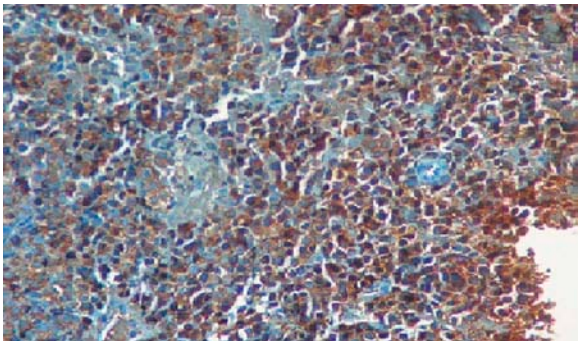

Figure 3. Immunohistochemical analysis showing liver plasmacytoma of IgA-kappa type.

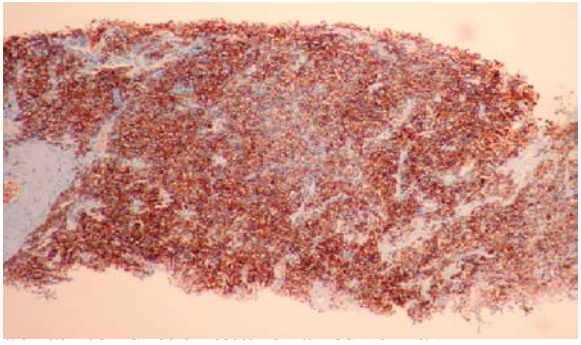

Figure 4. Immunohistochemical analysis showing strong membranous positivity of CD138.

Discussion

Plasmacytoma is an immunoproliferative, monoclonal disease and is classified as non-Hodgkin's lymphoma. Extramedullary plasmacytoma is a rare plasma cell neoplasm, constituting approximately 4% of all plasma cell neoplasms [4,5]. It may present as the sole manifestation of clonal plasma cell disease, as a metastasis from another extramedullary plasmacytoma, as solitary plasmacytoma of the bone, or as a consequence of multiple myeloma. It can be difficult to distinguish these plasma cell tumors from other neoplasms and reactive plasma cell proliferations [6]. Multiple myeloma presenting as hepatic space-occupying lesion (SOL), particularly as hyper-vascular SOL, similarly to hepatocellular carcinoma, is a rare presentation. Its etiopathogenesis is still unclear but it may be related to neoangiogenesis via vascular endothelial growth factor through its paracrine or autocrine mechanism [6–8].

Extramedullary plasmacytoma represents approximately 3% of all plasma cell neoplasms [9,10]. Although extramedullary plasmacytoma can appear in the whole body, almost 90% occur in the upper aerodigestive tract, and 17.8% in the gastrointestinal tract, urogenital tract, skin, lungs, and breasts (in that order) [11]. The diagnosis of EMP requires the absence of bone marrow plasmacytosis, a normal bone survey, and plasma-cell infiltration only at the site of extramedullary plasmacytoma[12].

Hepatic plasmacytoma has been reported to be hypoechoic on ultrasound, homogeneously hypodense with heterogeneously progressive enhancement on CT [13]. In our patient, several hepatic lesions were discovered by CT scan. A CT-guided biopsy of those lesions showed a monoclonal proliferation of plasma cells positive for the light chain detected by immunohistochemistry.

The histological diagnosis of plasmacytoma of the liver should be quite simple if the tumour is composed of sheets of well differentiated plasma cells. The differential diagnosis is limited to the plasma cell-rich variant of the inflammatory pseudotumours of the liver. The absence of spindle cells, histiocytes and lymphocytes between the plasma cells is suggestive of plasmacytoma [14]. With immunohistochemistry and molecular studies, the monoclonal nature of these tumours can be shown easily. Though, if the tumour is composed of rather poorly differentiated plasmablasts, then a distinction should be made from other high-grade B-cell lymphomas which also show light chain restriction and clonal immunoglobulin heavy chain rearrangements. The reported case is a confirmed kappa light chain multiple myeloma with liver plasmacytoma [15].

Conclusions

In conclusion, we presented a large and multiple lesion primary extramedullary plasmacytoma of the liver. Although the imaging findings of extramedullary plasmacytoma of the liver are nonspecific, differential diagnosis is required when a liver mass is present. The detection of a space occupying lesion in the liver in our patient was incidental and confirmed by cytology. Extramedullary appearances (still not that common) should be considered and multiple myeloma should be reserved as a widespread differential diagnosis of SOL of the liver. Biopsy was necessary to reach the final diagnosis. It is important to differentiate plasmacytoma from other hepatic metastases.

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Experimental & Clinical
Hepatology

Received: 2012.08.31
Accepted: 2012.09.20
Published: 2012.10.05

Suboptimal response to lamivudine therapy in nucleoside analogues (NUCs)-naïve patients with chronic hepatitis B – single-center data from clinical practice in Poland

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
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Source of support: Departmental sources

Summary

Background: Until 2010, lamivudine (LAM) was the only nucleoside analogue (NUC) available in Poland for the therapy of naïve patients with chronic hepatitis B (CHB). Currently, it is still the standard of care for HBeAg-negative patients. The aim of this study was to analyze outcomes and factors correlating with viral response (VR) to LAM therapy and resistance rate in a real-life setting.

Material/Methods: Data from CHB-infected patients treated with LAM was retrospectively analyzed. Eligibility criteria for analysis in the study were: more than 18 years of age, HBV monoinfection, NUC-naïvety, LAM therapy beginning after the year 2007, and LAM therapy lasting at least 12 weeks. VR was calculated at weeks 24, 48, 72, and 96 according to HBeAg-status, baseline HBV DNA, clinical diagnosis, age, and gender.

Results: Ninety-eight patients (69 males, 29 females, mean age of 44 years, 26 patients with cirrhosis) were selected according to study design criteria. Unfavorable response rates were observed in patients with intermediate and high baseline HBV DNA in both HBe-positive and HBe-negative groups. VR rates were correlated with baseline HBV DNA at weeks 24 (p=0.02) and 48 (p=0.006). There was no statistically significant correlation between viral response rate and diagnosis, age, and gender. The cumulative rate of HBsAg loss was 1.8%. The cumulative rate of HBeAg loss was 29.2%.

Conclusions: This study showed suboptimal response to LAM therapy in NUC-naïve patients with CHB, regardless of HBeAg-status.

Key words: **chronic hepatitis B • lamivudine • therapy • response**

Full-text PDF: http://www.expclinhep.com/fulltxt.php?ICID=883496

Word count: 914
Tables: 4
Figures: –
References: 8

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Background

Several drugs are available for the treatment of chronic hepatitis B (CHB): conventional interferon (IFN) alpha, pegylated IFN alpha, and nucleoside/nucleotide analogues (NUC). NUCs are divided into three classes: L-nucleosides (lamivudine, telbivudine, and emtricitabine), deoxyguanosine analogues (entecavir), and acyclic nucleoside phosphonates (adefovir and tenofovir). A 48-week course of pegylated IFN alpha is recommended in European guidelines for HBeAg-positive naïve patients with the best chance of HBe seroconversion. Pre-treatment factors predictive of HBe seroconversion are: low viral load (HBV DNA below 10^7 IU/mL), high serum ALT levels, and high activity scores on liver biopsy (at least A2). Treatment with NUCs for 24–48 weeks is available for HBeAg-positive patients who develop HBe seroconversion. Long-term treatment with NUCs is recommended for HBeAg-positive patients who have not developed HBe seroconversion and in all HBeAg-negative patients. According to EASL (European Association for the Study of the Liver) experts, all these treatment strategies should use the most potent available agents with the highest genetic barrier (entecavir or tenofovir) to rapidly reduce serum HBV viremia to undetectable levels and avoid development of HBV resistance [1].

Until 2010, lamivudine (LAM) was the only NUC available in Poland for the therapy of naïve patients with CHB. Currently, it is still the standard of care for HBeAg-negative patients. Polish experts recommend pegylated IFN alpha 2a and entecavir or tenofovir as a first-line CHB monotherapy [2]. However, according to the recent Polish Ministry of Health guidelines, the first-line treatment is monotherapy with pegylated IFN alpha 2a. NUCs are allowed in the case of contraindications to IFN alpha use or previous IFN alpha therapy failure. Adefovir, entecavir, or tenofovir are recommended for the therapy of HBeAg-positive patients and lamivudine for HBeAg-negative patients [3].

The aim of this study was to analyze outcomes and factors correlating with viral response (VR) to LAM therapy and resistance rates in a real-life setting.

Material and Methods

Data from CHB-infected patients treated with LAM was retrospectively analyzed. Eligibility criteria for analysis were: more than 18 years of age, HBV monoinfection, NUC-naïvety, LAM therapy beginning after the year 2007 (the year when the national therapeutic program for CHB was introduced), and LAM therapy lasting for at least 12 weeks. HBV DNA was measured at weeks 24, 48, 72, and 96 according to HBeAg-status, baseline HBV DNA (low $\leq 10^4$ copies/mL, intermediate $=10^4$–10^8 copies/mL, high $\geq 10^8$ copies/mL), clinical diagnosis, age, and gender.

Table 1. Baseline characteristics of patients.

Male N (%)	69	(70.4)
Age (mean, range, yrs.)	44	(18–83)
Clinical diagnosis N (%)		
Chronic hepatitis	72	(73.5)
Cirrhosis	26	(26.5)
Hepatocellular carcinoma	3	(3.1)
ALT >ULNR* N,%	81	(82.6)
HBeAg-negative N (%)	60	(61.2)
HBV DNA total, median, copies/mL	7.17×10^3	
HBV DNA HBeAg-pos., median, copies/mL	5.77×10^6	
HBV DNA HBeAg-neg., median copies/mL	4.04×10^3	

* Upper limit of normal range.

Statistical analyses

Statistical analyses were performed using logistic regression and Cochran-Armitage, chi-squared, Mann-Whitney, Kruskal-Wallis, and Fisher's exact tests.

Results

Ninety-eight CHB-infected patients treated with LAM were selected according to study design criteria. Table 1 presents baseline characteristics of the studied patients. In Table 2, baseline HBV DNA strata are shown. VR rates, serological outcomes, and ALT outcomes are summarized in Table 3. The rate of viral response (HBV DNA-negative) according to the baseline HBV DNA strata (low, intermediate and high) is presented in Table 4.

VR rates correlated with baseline HBV DNA at weeks 24 (p=0.02) and 48 (p=0.006) for low, intermediate, and high HBV DNA. There was no statistically significant correlation between viral response rate and diagnosis, age, and gender. The cumulative rate of HBsAg loss was 1.8%. The cumulative rate of HBeAg loss was 29.2%.

Discussion

In this study, the majority of CHB-infected patients are males with low baseline serum HBV viremia and increased ALT

Table 2. Baseline HBV DNA strata.

HBV DNA range		HBeAg-neg	HBeAg-pos	Overall group
Low	HBV DNA $<10^4$ c/mL N (%)	43 (71.7)	10 (26.3)	53 (54.1)
Intermediate	HBV DNA 10^4–10^8 c/mL N (%)	17 (28.3)	16 (42.1)	33 (33.7)
High	HBV DNA $>10^8$ c/mL N (%)	0 (0.0)	12 (31.6)	12 (12.2)

Table 3. VR rates, serological outcomes, and ALT outcomes in studied populations.

	Week 24 N=98	Week 48 N=98	Week 72 N=79	Week 96 N=57
VR* overall population %	38.8	40.8	48.1	42.1
VR* in HBeAg-negative pts. %	58.3	56.7	52.1	45.5
VR* in HBeAg-positive pts. %	7.9	15.8	42.0	37.5
HBsAg loss n (%)	0 (0)	0 (0)	0 (0)	1 (1.8)
HBeAg loss n (%)	4 (11.8)	6 (15.8)	8 (25.8)	7 (29.2)
Reseroconversion HBeAg (-) →HBeAg (+) n (%)	0 (0)	1 (1.7)	0 (0)	0 (0)
ALT ≤ULNR** n (%)	68 (69.4)	72 (73.5)	64 (81.0)	44 (77.2)

* Viral response: undetectable HBV DNA; ** upper limit of normal range.

Table 4. Rate of viral response (HBV DNA negative) according to the baseline HBV DNA strata (low, intermediate and high).

Baseline HBV DNA	Week 24 N=98	Week 48 N=98	Week 72 N=79	Week 96 N=57
HBV DNA $<10^4$ c/mL N (%)	47.2	50.9	54.5	41.7
HBV DNA 10^4–10^8 c/mL N (%)	36.4	36.4	41.4	50.0
HBV DNA $>10^8$ c/mL N (%)	8.3	8.3	33.3	0.0
P value	p=0.02	p=0.006	p=0.186	p=0.616

levels. A similar profile of CHB patients was recently shown in Germany [4]. Moreover, our virological data is consistent with current observations from China. Wu et al. investigated the HBV clinical virological characteristics of CHB infection in 1,572 Chinese patients of different age groups. Consequently, in the group of patients over 40 years of age, they observed significantly more reactivation cases than in any other group [5].

In this study, more than 60% of the studied population was post-HBe seroconversion. This tendency has already been observed in many countries because of aging of the HBV-infected population [6,7]. HBe-negative CHB is characterized by periodic reactivation with a pattern of fluctuating HBV DNA and ALT levels. Moreover, in this phase of HBV infection, low rates of prolonged spontaneous disease remission are observed [1].

The other disturbing trend in our population was the high percentage (almost 27%) of cirrhotic naïve patients. It has been shown that entecavir and tenofovir are the best therapeutic option for HBV-infected cirrhotic patients because of their high efficacy, safety, and observed improving in fibrosis score during treatment [8]. Unfortunately, in current Polish guidelines, LAM is still the first-line treatment for HBeAg-negative cirrhotic patients [3].

In this study, viral response to LAM therapy was suboptimal in patients with CHB, regardless of HBeAg-status. Negativity of serum HBV DNA was achieved in less than half of the patients at each point of the analyzed treatment period. It has been shown that the VR rate at one year post-treatment with LAM is 36–40% and 72% for HBeAg-positive and HBeAg-negative patients, respectively [1]. In week 48 of observations, the VR rate was about 57% in HBeAg-negative subjects; however, in the HBeAg-positive population, the VR rate was less than 20%. As a result, our data confirmed that LAM should not be the first-line treatment for either the HBeAg-positive or the HBeAg-negative population in Poland. Furthermore, viral efficacy depended on the baseline HBV DNA level. Unfavorable response rates were observed in patients with intermediate and high baseline HBV DNA in both HBeAg-positive and HBeAg-negative groups.

Conclusions

In conclusion, this study showed the suboptimal response to LAM therapy in NUC-naïve patients with CHB, regardless of HBeAg status.

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Experimental & Clinical
Hepatology

Received: 2012.08.31
Accepted: 2012.09.10
Published: 2012.10.11

Acute hepatitis C in patients with advanced malignancies

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
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Source of support: Departmental sources

Summary

Background: Hepatitis C virus (HCV) infection in patients with advanced malignancies treated with cytostatics differs from HCV infection in general population due to additional factors connected with liver disease, like immunosuppression and drug-induced hepatotoxicity.

Aim: To describe the effect of nosocomial HCV infection on the clinical course of hepatitis, efficiency of antiviral treatment and outcome in patients with advanced cancer.

Material/Methods: A cohort of 24 women (mean age 47 years) with ovarian or breast cancers and acute HCV infection was studied. The control group consisted of 10 non-neoplastic patients infected with HCV during hospitalization at the same gynecology department. The diagnosis of acute hepatitis C (AHC) was based on both anti-HCV by EIA 3.0, HCV-RNA positive tests and elevated alanine aminotransferase (ALT). We studied: clinical symptoms, liver changes on ultrasonography, serum activity of ALT, γ-glutamyltransferase (GGT), alkaline phosphatase and bilirubin level. Sixteen patients were treated with interferon α (IFN). Efficacy and tolerability of the therapy were assessed in both groups.

Results: Spontaneous clearance of HCV was observed in 8% of patients with advanced malignancies. Sustained virological response was achieved in 51.7% of patients with cancer treated with pegylated IFN and ribavirin in the early phase of chronic hepatitis C. No severe liver damage was observed in patients with AHC treated with chemotherapy. No differences were found with respect to the clinical course of AHC between women with advanced cancer and control groups. Significantly lower ALT (p=0.02) and higher GGT (p=0.006) activities were observed in patients with advanced malignancies treated with cytostatic drugs in comparison to controls.

Conclusions: Advanced malignancies and chemotherapy do not exclude the possibility of spontaneous HCV clearance and favourable virological response of majority of patients treated with IFN. Chemotherapy of patients with advanced cancer in the course of AHC was not a life-threatening condition.

Key words: **hepatitis C • nosocomial infection • breast and ovarian cancer • chemotherapy • interferon alpha treatment**

Full-text PDF: http://www.expclinhep.com/fulltxt.php?ICID=883501

Word count: 1692
Tables: –
Figures: 4
References: 21

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Background

Acute hepatitis caused by HCV infection (AHC) is rarely diagnosed and documented in comparison to acute HAV or HBV infections [1,2]. Changes in epidemiology of HCV infection with significant reduction of post-transfusion hepatitis resulted in a different clinical course of AHC [3–8]. Nosocomial HCV infections still remain an important cause of AHC [9–11].

The course of AHC in patients with advanced neoplastic disease and nosocomial AHC might be different. Cytostatic treatment in immunocompromised patients with advanced neoplastic disease can affect the outcome and course of AHC [12]. The decision of antiviral therapy might be difficult and AHC can affect the continuation of chemotherapy. Chemotherapy was introduced just before the onset of AHC and it was a potential risk of acute liver injury [13,14]. The studies of patients with malignancies treated with potentially hepatotoxic medicines are sparse but may appear helpful in understanding the course of the acute phase of HCV infection.

The aim of the study was to evaluate clinical symptoms, course, therapy and outcome of AHC in patients with advanced malignancies in comparison to the control group of HCV-infected patients without neoplastic disease.

Material and Methods

Thirty-four patients with AHC treated at the onco-gynecology department, 24 patients with neoplastic disease and 10 patients without malignancies were included in the study. The diagnosis of AHC was established by positive HCV-RNA (COBAS® TaqMan® HCV 2.0), increased activity of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) more than 10 times above normal levels before hospitalization, positive HCV antibodies by EIA 3.0 or confirmed seroconversion. Infections with HAV, HBV, CMV and EBV were excluded.

All patients with neoplastic disease were treated with chemotherapy before the onset of AHC or chemotherapy had to be continued after HCV infection was diagnosed.

There were no differences in age between the two groups of patients ($p>0.05$). Patients with breast and ovarian cancers were treated with cytostatic drugs as: paclitaxel derivates, cisplatine, topotecan, etoposide, vinorelbine, doxorubicine, bleomycin, cyclophosphamide, metothrexate and fluorouracil, according to the current oncological recommendation. All patients were examined with colposcopy and treated with intravenous infusions of drugs and 29 patients were operated on. The source of outbreak was not identified and could be related to unsafe medical procedures. All AHC cases were diagnosed in year 2003.

Patients were observed during the acute phase of HCV infection and for a prolonged time in protracted infection cases. The control group consisted of 10 patients hospitalized at the same onco-gynecology department but without malignancies.

The presumable time of HCV infection was established in 16 patients on the basis of incubation time lasting from the beginning of hospitalization at the onco-gynecology department to the appearance of symptoms or biochemical indices of liver injury. Clinical symptoms, abdominal sonographic changes, activity of ALT (ALAT/GPT Cobas), AST (ASPAT/GOT Cobas), γ-glutamyltransferase (GGT Cobas), alkaline phosphatase (ALP Cobas) and serum bilirubin levels were evaluated in both groups. Antiviral treatment of AHC consisted of interferon α 2b (Intron A, Schering-Plough Corp.) in doses of 5 million IU three times a week for 24 weeks, and in chronic hepatitis C (CHC): peginterferon α 2a (PegIntron, Schering-Plough Corp.) in doses of 1.5 mcg/kg/week for 48 weeks with ribavirin (Rebetol, Schering-Plough Corp.) in doses of 15 mg/kg per day or peginterferon α 2b (Pegasys, Roche Corp.) in doses of 180 mcg/week for 48 week with ribavirin (Copegus, Roche Corp.) in doses of 1000 mg per day if weight <75 kg or 1200 mg if ⩾75 kg, in 16 patients of both groups, fulfilling the inclusion criteria. The efficacy of antiviral treatment was evaluated. HCV genotype 1b (Versant® HCV Genotype 2.0 Assay) was detected in all treated patients.

Differences between the two groups in clinical symptoms such as dyspeptic and flu-like complaints, jaundice and sonographic changes of the liver were estimated by logistic regression. Simultaneous evaluation of all symptoms, taking into account the number of symptoms in every patient of each group, was carried out using a non-parametric Mann-Whitney U test. Average ALT, AST, GGT, ALP activity and bilirubin levels were estimated by a single-factor analysis of variance in ANOVA model. All p-values less than 0.05 in the two-tailed test were considered significant. All statistical analyses were performed using software Statistica v. 9.0 (StatSoft, Inc., Tulsa, Oklahoma, USA).

The study was carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association.

Results

No statistically significant differences were found between neoplastic patients and control group in the prevalence of dyspeptic and flu-like symptoms, elevated body temperature, jaundice and sonographic changes in the liver evaluated either separately (Figure 1) or in groups by Mann-Whitney U test (Figure 2).

Statistically significant differences were found in ALT ($p=0.0231$) and GGT ($p=0.0059$) activities in blood serum. ALT activity was lower and GGT activity was higher in patients with advanced cancer (Figure 3). Statistically significant differences in ALT/GGT ratio ($p=0.0003$) and ALT/ALP ratio ($p=0.0012$) were found between the groups, with p-value lower then for ALT, GGT and ALP differences.

Chemotherapy administered in the course of AHC affected ALT activity. It was described as a multiple phase chart with inflammatory exacerbations soon after infusions of cytostatics (Figure 4). Chemotherapy preceding the onset of AHC or continued during the course of AHC did not worsen liver damage and hepatic decompensation was not observed.

In the group of patients with cancer and HCV infection, spontaneous clearance of HCV was reported in 2 (8%) of them. One of the patients, who started treatment of AHC with a

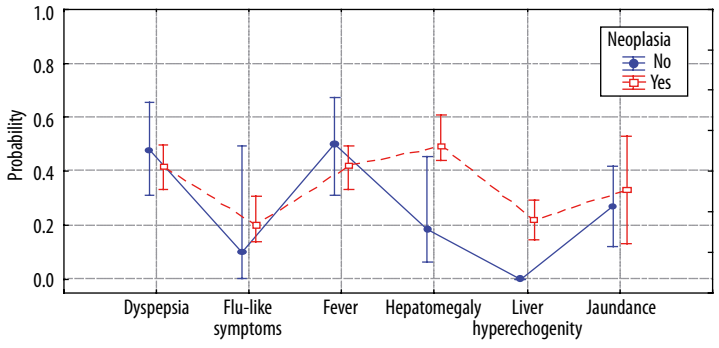

Figure 1. Clinical symptoms in patients with advanced gynecological malignancies and control group according to logistic regression model; $p>0.05$.

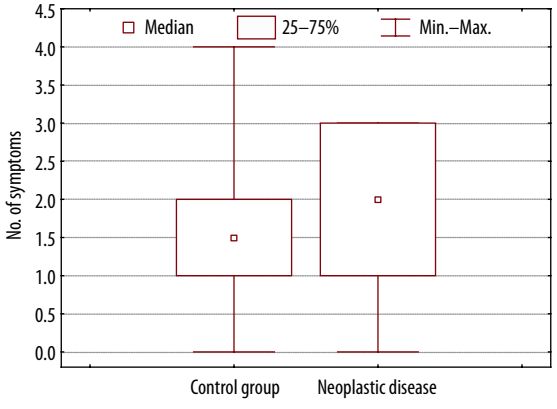

Figure 2. Clinical symptoms in patients with neoplastic disease and control. Non-parametric Mann-Whitney U test; $p>0.05$.

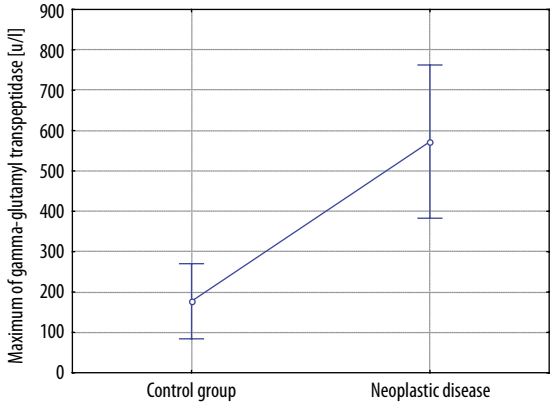

Figure 3. Activity of γ-glutamyl transpeptidase in the serum of patients with neoplastic disease and control group. ANOVA model; $p=0.0003$.

standard IFN monotherapy, did not achieve positive response. Seven patients were treated with pegylated interferon α plus ribavirin during CHC, and SVR was achieved in 4 (51.7%) of them. In 2 patients with early viral response (EVR), treatment was discontinued because of neoplastic disease relapse.

The achieved results of therapy were not compared with the control group (patients without malignancies), because 4

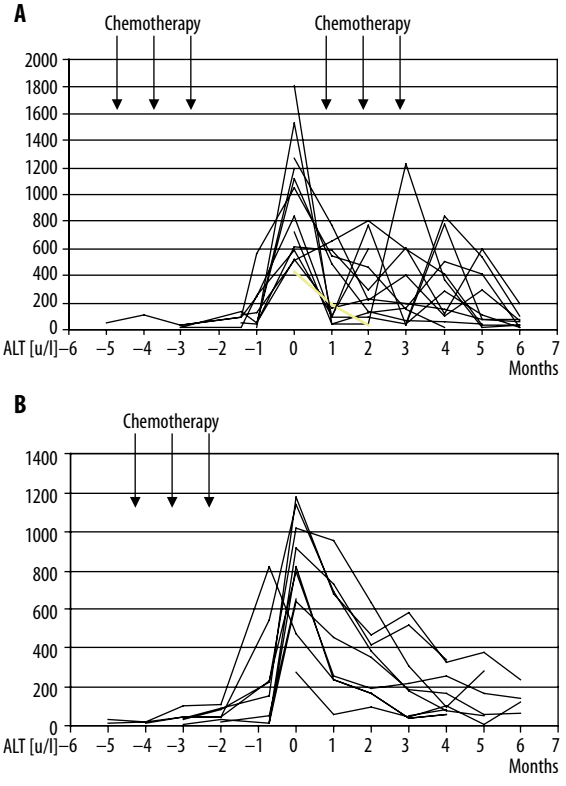

Figure 4. Alanine aminotransferase (ALT) activity in the serum of patients with advanced neoplastic disease treated with chemotherapy in the course of AHC (**A**) and without (**B**). Month 0 – onset of AHC, "minus" – months before onset of AHC.

patients started effective standard IFN monotherapy within 12 weeks from AHC diagnosis. Too early initiation of therapy in the control group was probably responsible for the fact that spontaneous eradication of HCV was observed only in one person. Frequency of spontaneous, as well as therapy-related eradication of HCV among oncologic patients was presented in comparison with data from previous studies.

Discussion

Neoplastic disease coexisting with AHC did not influence the type and frequency of symptoms of acute-phase HCV infection in the investigated group. Administration of chemotherapy in patients with malignancy and AHC was connected with exacerbation of symptoms, particularly dyspeptic.

Prevalence of the symptoms in our study was higher than described by other authors [1,2,8,15]. Dyspeptic syndrome and low-grade fever were observed in half of the patients, jaundice in 30% and flu-like symptoms in 20% of patients.

Jaundice in both investigated groups was of low intensity, and bilirubin level was lower than 150 umol/L. Among patients with malignancy who received chemotherapy before or in the course of AHC, symptoms of liver failure were not observed.

There was no difference in the estimated incubation period of HCV infection between the investigated neoplastic patents with AHC and the control group.

Hepatomegaly and liver hyperechogenicity on ultrasound were detected more often in patients with cancer, but without statistical significance (Figure 2).

Analysis of biochemical results showed significantly lower ALT and higher GGT activities in serum of patients with advanced malignancies. There was a tendency for an increase in ALP in those patients in comparison to the control group. Both ALT/GGT and ALT/ALP ratios enabled the best differentiation of the investigated groups. Lower ALT activity as well as its multiple-phase chart in neoplastic patients corresponded to other results concerning lower probability of HCV eradication during AHC and higher prevalence of protracted acute phase of the disease in those patients [3].

Those biochemical data suggest a hepatotoxic effect of chemotherapy in patients with AHC and neoplastic disease. Liver injury caused by cytostatic drugs resembled the direct hepatotoxic pattern with increased ALT and AST activities which appeared with anticipated regularity shortly after exposition to the drug and disappeared after discontinuation of chemotherapy. Those changes were in contrast with clinical symptoms of an earlier phase of malignant disease before onset of AHC when the effect of chemotherapy on liver tests in the same patients was not important (Figure 4). Such observations suggest an increased susceptibility of the liver to cytotoxic effects of drugs in AHC.

No severe liver damage classified as acute liver failure was observed. Acute liver failure rarely complicates the course of AHC, although some cases of severe liver injury in neoplastic patients treated with chemotherapy were reported [13,14]. Decision to start chemotherapy in a patient with AHC and neoplastc disease is difficult because of possible hepatotoxicity. Based on previous observations, high GGT activity in patients with advanced neoplastic disease may suggest some role of microsomal oxidation and oxidative stress in the mechanism of liver injury in patients with AHC on chemotherapy [16].

Spontaneous eradication of HCV in patients with neoplastic disease was observed in 8% patients, i.e. less frequently than in the general population [1,3,6,17]. However, this corresponds with results from severely immunocompromised patients or with post-transfusion AHC [4,5,7,18]. During AHC, only one oncologic patient was treated with interferon α because of contraindications connected with the malignant disease.

Despite the observed low prevalence of spontaneous eradication of HCV in the patients with malignancy, therapy with pegylated interferon α plus ribavirin in the early phase of CHC appeared to be effective in 57.1% of patients. In contrast to the low spontaneous eradication of HCV, the prevalence of sustained virological response was the same as in HCV-infected patients in the general population without malignancies [19–21].

Conclusions

Apart from more serious dyspeptic symptoms, the clinical course of AHC in patients with an advanced neoplastic disease does not differ from the one in a non-neoplastic population. Biochemical tests in neoplastic patients on chemotherapy such as lower mean ALT and higher GGT activities might suggest different mechanisms of liver damage in the patients. Chemotherapy in patients with AHC was not associated with a higher risk of severe liver injury. Neither neoplastic disease nor cytostatic therapy exclude the possibility of spontaneous eradication of HCV in the early phase of infection or viral response in patients with chronic hepatitis C treated with antiviral therapy.

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Experimental & Clinical
Hepatology

Received: 2012.09.12
Accepted: 2012.09.12
Published: 2012.10.16

Quantitative Shear Wave Ultrasound Elastography: A new, non-invasive method for liver fibrosis staging in patients with chronic hepatitis C

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: Departmental sources

Summary

Background: The extent of liver fibrosis influences both treatment and surveillance strategies in chronic hepatitis C. However, accurate histological tissue evaluation is not devoid of limitations and there have been attempts to find a substitute for liver biopsy as the gold standard for detecting hepatic fibrosis. Ultrasound elastography is a recently introduced technique that uses ultrasound to quantify tissue stiffness by measuring the speed of shear wave propagation.

The aim of this study was to compare data obtained using ultrasound-based elastography to liver biopsy results for the assessment of fibrosis in chronic hepatitis C.

Material/Methods: Twenty-seven patients (15 females; mean age 44±13 years) with chronic viral hepatitis C underwent liver biopsy and ultrasound-based elastogaphy examination. In addition, in all individuals, peripheral blood parameters, including aspartate aminotransferase, alanine aminotransferase, γ-glutamyl transpeptidase, total protein, albumin, α-fetoprotein and lipid profile, were measured. The Spearman's rank correlation coefficient, the Kruskal-Wallis and the Mann-Whitney tests were used in order to establish the relationship between the stage of liver fibrosis (METAVIR), blood test results and wave velocity scores.

Results: Percutaneous liver biopsy revealed no signs of fibrosis in four subjects (F0). Fibrosis was staged as mild (F1) in 13, moderate (F2) in 5 and severe (F3) in 3 cases, respectively, whereas cirrhosis (F4) was recognized in two instances. The mean wave velocity values according to the fibrosis stage were as follows: F0, 1.27 m/s; F1, 1.44 m/s; F2, 1.26 m/s; F3, 1.77 m/s; F4, 2.47 m/s. However, statistical analysis showed no correlation between liver biopsy results and wave velocity scores.

Conclusions: Although it seems that ultrasound elastography could find its place in clinical practice, especially in differentiating between cirrhosis and pre-cirrhotic stages of liver fibrosis, its value in accurate delineation of fibrosis stages requires further investigation.

Key words: **ultrasound elastography • transient elastography • chronic hepatitis C • tissue stiffness • liver fibrosis • percutaneous liver biopsy**

Full-text PDF: http://www.expclinhep.com/fulltxt.php?ICID=883505

Word count: 1605
Tables: 2
Figures: 2
References: 35

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Background

Epidemiology of hepatitis C

With an estimated 170 million persons infected worldwide [1–3], hepatitis C virus poses a serious medical problem. The prevalence of HCV infection in Poland is 1.5% [4–6] in the general population, equating to approximately 750 thousand anti-HCV-positive persons nationwide [4]. According to the National Institute of Public Health, the number of new infections totaled 1891 in 2009, yielding an incidence rate of 5 cases per 100 000 persons [7].

HCV is transmitted by percutaneous exposure to blood, contaminated blood products and medical equipment. At present, in Polish population there are two age groups in which different modes of transmission are predominant. Among those aged 30 or less, between 50% and 70% acknowledge illicit injection drug use [5,6,8].

In contrast, in older people, up to 90% of infections were healthcare-associated. Not surprisingly, the highest prevalence of infection is found among patients who give a history of repeated administration of blood products, e. g. patients with hemophilia treated with clotting factor concentrates produced before 1987 [9]. Although blood transfusions were once an important source of infection, since the introduction of routine donated blood screening in 1992, the transmission rate has declined to 1 in 1 million units transfused [6].

Furthermore, HCV can be acquired both vertically and sexually, the number of reported cases is, however, relatively low [6,10]. There have also been instances of transmission via occupational exposure to HCV-positive blood and there is an estimated 3% risk of infection per single needlestick injury [11].

Natural history

HCV is a single-strand, positive-sense RNA virus, classified within the family Flaviviridae. The virus can be divided into six major genotypes and over a hundred subtypes [3,11,12]. HCV infection rarely presents itself as acute hepatitis (on average, only one in ten patients develops clinical jaundice). Indeed, there is up to an 85% risk of persistent infection, with liver fibrosis as its inevitable sequel. Gradual changes in the lobular architecture lead eventually to liver cirrhosis, which occurs in up to one-fourth of patients within 20 to 30 years [10,12]. Several factors are believed to contribute to faster disease progression, including alcohol consumption, male gender, older age of acquisition and HBV coinfection. Once cirrhosis is established, there is an increased likelihood of hepatocellular carcinoma and end-stage liver disease development [11,12].

Liver biopsy

At present, liver biopsy is the gold standard for accurate staging of liver fibrosis. In Poland, according to the national chronic hepatitis C treatment guidelines, histological evidence of inflammation and fibrosis is required in order to administer interferon therapy [13]. Moreover, the degree of fibrosis correlates with the probability of achieving a sustained response to antiviral treatment. Nonetheless, percutaneous liver biopsy is an invasive procedure and therefore carries a risk of complications, such as pain, intrahepatic or subcapsular hematomas and transient hypotension, to name but a few [14]. What is more, in instances of severe clotting disorders, suspected hemangioma or in uncooperative patients, liver biopsy may be contraindicated. Other limitations of this technique include inter- and intra-observer variability in biopsy assessment and sampling errors [12,15–19].

Resulting from the need for a unified histological assessment system in chronic hepatitis C, a number of scoring schemes have emerged. One of the most commonly used is the METAVIR scoring system, which was also employed in this study. It incorporates two different scores for both grading, i. e. the activity of necroinfammatory process, as well as staging (the degree of fibrosis) of liver disease. The latter is classified into five categories, ranging from F0 to F4 (F0, no fibrosis; F1, portal fibrosis without septa; F2, portal fibrosis and few septa; F3, numerous septa without cirrhosis; F4, cirrhosis) [20].

Elastography

The problems associated with obtaining and interpreting liver biopsy specimens have prompted the search for alternative liver fibrosis assessment methods. Transient elastography (FibroScan®) is a newly developed technique for liver elasticity measurement which has shown promising results in a number of studies. It involves using an ultrasound probe to measure the velocity of a shear wave generated by a vibrator. The speed of wave progression increases with tissue stiffness [21–24]. However, owing to the fact that the device is relatively expensive and thus not readily available in clinical practice, a novel approach, using ultrasound-based transient elastography which can be performed with conventional probes during a routine sonography examination, has been extensively investigated. The advantages of this method are: no identified complications and contraindications, painlessness, instantaneous result, reproducibility and a large portion of the liver evaluated. On the other hand, in patients with significant necroinflammatory activity, steatosis, obesity or ascites liver stiffness measurements may be of limited utility [25–27].

Objective

The aim of this study was to compare data obtained using ultrasound-based elastography to liver biopsy results for the assessment of fibrosis in chronic hepatitis C.

Material And Methods

Twenty-seven patients (fifteen females) with chronic viral hepatitis C, hospitalized in the Department of Infectious Diseases and Hepatology between January and December 2010, were included in this study. Subjects averaged 44 years of age (range 21–66). In all patients viral hepatitis C was confirmed by the presence of HCV antibodies and HCV-RNA. Elastography was performed in the Department of Radiology at M. Skłodowska-Curie Regional Hospital, Szczecin, using the Siemens 3.5 MHz convex array ultrasound probe. The readings were taken from the right lobe of the liver through the intercostal space and the mean value of five successful measurements was considered representative. Results are expressed in

Table 1. Liver function panel of 27 patients with chronic hepatitis C.

	AST [IU/ml]	ALT [IU/ml]	GGTP [IU/ml]	TP [g/dl]	ALB [g/dl]	AFP [IU/ml]
Mean	70.3	50.3	91.9	7.1	4.2	7.1
Minumum	21.0	20.0	7.0	3.6	3.3	1.0
Maximum	162.0	227.0	539.0	8.7	4.9	48.3
Standard deviation	±50.8	±33.7	X	±1.0	±0.4	X

AST – aspartate aminotransferase; ALT – alanine aminotransferase; GGTP – γ-glutamyl transpeptidase; TP – total protein; ALB – albumin; AFP – α-fetoprotein.

Table 2. Blood lipid values.

	Total cholesterol [mg/dl]	HDL [mg/dl]	LDL [mg/dl]	Triglycerides [mg/dl]
Mean	191	63	97	106
Minumum	103	29	27	44
Maximum	445	87	292	233
Standard deviation	±76.7	±17.6	±50.7	±52.5

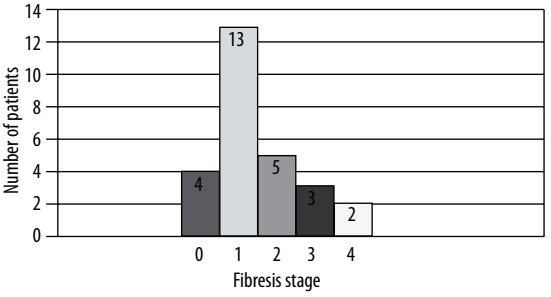

Figure 1. Patient distribution according to fibrosis stage (METAVIR).

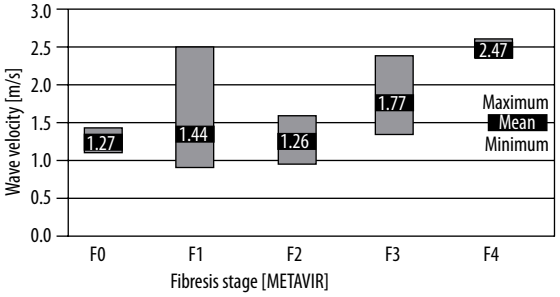

Figure 2. Distribution of wave velocity scores according to METAVIR fibrosis stage.

meters per second. Percutaneous liver biopsy followed within a 24-hour period. Liver tissue specimens were evaluated by the same pathologist, according to the METAVIR scoring system. In all individuals peripheral blood parameters, including aspartate aminotransferase, alanine aminotransferase, γ-glutamyl transpeptidase, total protein, albumin, α-fetoprotein and lipid profile, were measured. Statistical analysis was performed using the Spearman's rank correlation coefficient, the Kruskal-Wallis or the Mann-Whitney tests, as appropriate.

Results

General characteristics of the study group, including hepatic function tests and lipid profile values are presented in Tables 1 and 2, respectively. No statistical correlation was found between elastography measurements and blood test results. With regard to percunaneous liver biopsy results, no signs of fibrosis were revealed in four subjects (F0). Fibrosis was staged as mild (F1) in 13, moderate (F2) in 5 and severe (F3) in 3 cases, whereas cirrhosis (F4) was recognized in two instances (Figure 1). The overall average wave velocity in the study group was 1.49 (±0.51) m/s. Figure 2 demonstrates the distribution of wave velocity scores among patients with different METAVIR fibrosis stages, as well as the average score for each group. Among the individuals without evidence of fibrosis, the mean wave velocity equaled 1.27 m/s (range 1.1–1.47 m/s) and was almost exactly the same as in the moderate fibrosis group (1.26, range 0.97–1.62 m/s). There was only a slight increase in the average score in patients with mild fibrosis (1.44, range 0.96–2.5m/s), whereas the readings in the severe fibrosis group were, on average, significantly higher (1.77, range 1.35–2.43 m/s). Although the average score in cirrhotic patients almost doubled, compared to the subjects with histologically normal liver tissue (average 2.47 m/s, range 2.35–2.58 m/s), the fact that there were only two patients in the former group requires consideration. Moreover, statistical analysis proved no relationship between the wave velocity scores obtained with ultrasound-based elastography and liver biopsy results.

Discussion

Despite the fact that the wave velocity does seem to increase with hepatic disease progression, the comparison of mean values for each fibrosis stage reveals a significant overlap in the readings from patients with no, mild and moderate fibrosis (F0–F2), and this trend is consistent with other findings [23–24,26]. Although transient elastography was proven to have a high diagnostic accuracy for differentiation between milder (F1–F2) and more advanced (F3–F4) fibrosis, the cut-off values are still to be determined [23,24,26,29].

The search for liver biopsy substitute does not only focus on transient elastography. Currently, apart from imaging modalities, serological fibrosis markers have also come under scrutiny. These range from simple (APRI) to more complex algorithms (ELF, FibroTest) [15,28–30]. Other studies have shown a strong relationship between both elasticity scores as well as serum markers and liver fibrosis, as determined by histological examination. Nonetheless, the best diagnostic accuracy was obtained by combining these two methods [22,31–33]. It therefore seems that elastography, used in conjunction with blood tests, can further improve the delineation between different fibrosis stages.

However, it should be taken into consideration that liver biopsy, being the reference method for the evaluation of non-invasive tests, does have certain inherent methodological limitations, which make this gold standard somewhat flawed. Not only are the inter- and intra-observer variability being well documented, but also, stemming from the fact that a biopsy specimen represents only 1/50 000 of the liver, sampling variability seems inevitable [24,33].

On the other hand, liver biopsy reveals more detailed information regarding associated conditions that might affect disease progression or patient management, such as alcoholic liver disease, autoimmune hepatitis, haemochromatosis, nonalcoholic steatohepatitis, steatosis or biliary obstruction and in these instances should not be disregarded [34,35].

Conclusions

Despite the small number of subjects included in this study, our findings suggest that elastography might prove useful in approximate evaluation of the extent of liver fibrosis, and more importantly, in differentiating between mild and severe hepatic fibrosis. Furthermore, owing to its undeniable advantages over repeated liver biopsy, ultrasound elastography could become an invaluable diagnostic tool for monitoring patients with expected rapid disease progression, e.g. liver transplant recipients. Nevertheless, it should be stressed that liver biopsy remains the best standard available in accurate chronic liver disease assessment and elastography as its surrogate requires further investigation.

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Experimental & Clinical
Hepatology

Received: 2012.11.30
Accepted: 2012.12.03
Published: 2012.12.18

The activity of serum and urinary α-L-fucosidase in binge drinking

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B Data Collection
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Source of support: Departmental sources

Summary

Background: Alpha-fucosidase (FUC) is a lysosomal exoglycosidase involved in degradation of fucose-containing oligosaccharides, glycoproteins and glycolipids. Increased activities of FUC were earlier reported in the sera of alcohol dependent patients with alcoholic cirrhosis. The objective of this study was to determine serum and urinary activity of α-L-fucosidase after acute ethanol intoxication (binge drinking).

Material/Methods: Activity of α-fucosidase was determined in serum and urine samples of eight healthy binge drinkers collected before acute ethanol ingestion (120–160 g) as well as 2, and 5 days thereafter using a colorimetric method.

Results: After a binge drinking session, activity of FUC in the serum did not significantly increase, whereas urinary activity of FUC increased significantly on the fifth day after drinking. We found a positive correlation between serum FUC and serum aspartate aminotransferase levels measured on the 5th day after drinking and an inverse correlation between urinary FUC and serum alanine aminotransferase concentrations on a 2nd day after drinking. The cut-off value of 60 pKat/ml for urinary FUC activity demonstrated good sensitivity (87.5%) and specificity (87.5%).

Conclusions: Binge drinking-induced increase in urinary activity of FUC is associated with liver dysfunction during alcohol consumption and can be detectable even five days thereafter.

Key words: α-L-fucosidase • serum • urine • aminotransferase

Full-text PDF: http://www.expclinhep.com/fulltxt.php?ICID=883661

Word count: 1150
Tables: 1
Figures: 2
References: 13

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Background

Alpha-fucosidase (FUC) is a lysosomal exoglycosidase involved in degradation of fucose-containing oligosaccharides, glycoproteins and glycolipids [1]. Increased activities of lysosomal enzymes such as β-hexosaminidase, α-fucosidase, and α-mannosidase, were earlier reported in the sera of patients suffering from alcohol dependence, especially in case of coexisting liver damage [1–3]. Urinary product of FUC degradation, L-fucose, was described as a potential marker of alcoholic liver disease [3]. It was also described that acute ethanol administration in rats significantly reduced incorporation of ^{14}C-fucose into serum transferrin [3]. Generally, altered metabolism of glycoproteins in alcoholic liver disease might to be due either to altered glycoconjugate biosynthesis, modification, transport and secretion, or to the processes of elimination and catabolism that also occur in the extracellular matrix [4–7]. Increase in salivary β-hexosaminidase, α-fucosidase, and β-glucuronidase as well as serum and urinary β-hexosaminidase was reported after a binge drinking session (*binge drinking* – consumption of alcohol leading to intoxication, drinking more than 5 standard drinks on one occasion) [8–10].

To the best of our knowledge, this is the first study to examine the activity of α-fucosidase in healthy volunteers after acute ethanol intoxication (binge drinking session).

Material and Methods

Eight non-smoking men (aged 22–31 years, 27.0±2.5; BMI 25.0±1.7), who were not receiving any medications, participated in the study. Prior to the experiment, all volunteers were clinically verified to be in good general health. All men were infrequent binge drinkers (reported bingeing 1–11 times per year and/or 1–2 episodes in the past month), who had abstained from alcoholic beverages and drugs for 10 days before the experiment. Participants stayed at home during the drinking session under supervision of sober friends and a physician, who helped to verify the quantities and the time when drinking stopped. During sessions of alcohol consumption (7 p.m. to 1 a.m.) participants ingested 120–160 g of ethanol (12–16 standard drinks) such as 40% vodka (2.0 g/kg of body weight; ranging from 1.42 to 2.5 g/kg) together with light meals and fruit juice (excluding grapefruit juice). Such amounts of alcohol are common in spirit-drinking countries, including Poland, provoking a tolerable but severe intoxication [8]. The study was approved by the local Bioethical Committee of the Medical University of Bialystok, Poland. Informed written consent was obtained from all participants after the explanation of the nature, purpose and potential risks of the study. The subjects were deprived of food and beverages, except water, for 2 h before sample collection. The sets of blood and urine samples were collected (before drinking – BD, on the second day after drinking, and on the fifth day after drinking) and subsequently centrifuged to remove cells. Supernatants were divided into 200-μL portions, frozen and kept until analysis.

Activity of FUC in supernatants of serum and urine was determined in duplicates by the colorimetric determination of p-nitrophenol released from p-nitrophenyl-β-D-fucopyranoside (Sigma, USA) [11].

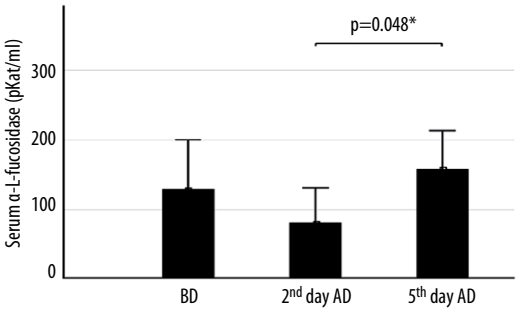

Figure 1. Activity of serum α-L-fucosidase before drinking (BD), on the second day after drinking (2nd day AD), and on the fifth day after drinking (5th day AD).

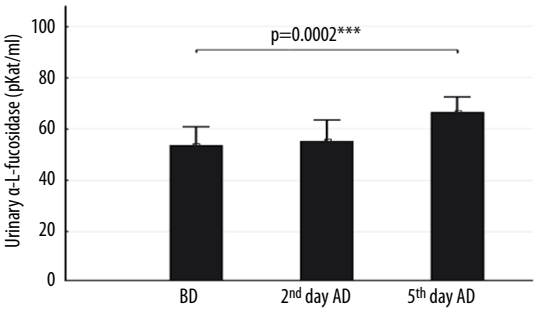

Figure 2. Activity of urinary α-L-fucosidase before drinking (BD), on the second day after drinking (2nd day AD), and on the fifth day after drinking (5th day AD).

Activity of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were determined according to standard laboratory techniques (EMAPOL reagents, Poland).

Statistical analysis

Statistical analysis was performed using Statistica 10.0 (Statsoft, Cracov, Poland) software. Changes in GLU activity across time were analyzed by Friedman analysis of variance (ANOVA) and Kendall concordance. For comparison between aminotransferase levels, Wilcoxon matched pair test was used. Spearman's rank correlation coefficient was used to measure the statistical dependence between two variables. Calculations of specificity and sensitivity were performed using STATISTICA's Rapid Deployment of Predictive Models software. Statistical significance was defined as $P<0.05$.

Results

As shown by Figure 1, after a binge drinking session the activity of FUC (pKat/ml) in serum did not significantly increase, whereas urinary activity of FUC increased significantly on the fifth day after a binge drinking session (p=0.0002***) (Figure 2).

AST activity was significantly lower on the fifth day after a binge drinking session compared to the second day (p=0.027*) (Table 1). There were no significant differences in ALT activity between the second and the fifth day following binge drinking event (p=0.402).

Table 1. The effect of binge drinking on the serum activity of aspartate (AST) and alanine (ALT) aminotransferases on teh second day after drinking (2nd day AD), and on the fifth day after drinking (5th day AD).

Variable	2nd day AD	5th day AD	*p* value
AST	31±10	26±10	0.027*
ALT	38±11	36±9	0.402

Values are expressed as mean ± SD; n=8; *p* Value <0.05 considered statistically significant; *p* Value: 2nd day AD to 5th day AD.

We did not find any correlations between amounts of alcohol and FUC, AST, or ALT at any time point after drinking. We found a positive correlation between serum FUC and serum AST on the 5th day after drinking (r=0.842, p=0.017*), and an inverse correlation between urinary FUC on the 2nd day after drinking and serum ALT (r=–0.796, p=0.032*).

The cut-off value of 60 pKat/ml for urinary FUC activity showed good sensitivity (87.5%) and specificity (87.5%).

Discussion

It was reported that alcohol-induced elevation in activity of lysosomal enzymes (including glycohydrolases e.g. FUC) in the serum and urine were due to increased biosynthesis, increased lysosomal membrane permeability and leakage of enzymes into cellular cytoplasm, intercellular space and body fluids, delayed removal of enzymes from the fluid, impaired glycosylation and trafficking of lysosomal hydrolases into the organelles, enhanced synthesis of the enzyme by activated leucocytes or leakage from damaged cells [4–9]. Beside ethanol oxidation products such as acetaldehyde and reactive oxygen species (ROS) generated during alcohol consumption, non-oxidative metabolites of ethanol (e.g. fatty acid ethyl esters; FAEEs), ethanol-water competition mechanism as well as decreased oxidized/reduced nicotinamide adenine dinucleotide ratio (NAD+/NADH) and resulting reduction in adenosine triphosphate (ATP) synthesis might potentially be involved in liver tissue damage (for review, see Waszkiewicz et al. [5]). All of the abovementioned mechanisms of alcohol toxicity may destabilize/damage lysosomal and cellular membranes, increasing membrane fragility, thus allowing for leakage of hydrolases [4,10]. As alcohol intoxication increases diuresis and urinary clearance of creatinine [12], increased activity of FUC in urine, even five days after drinking, might be the result of high diuresis. Urinary markers can be detectable much longer than serum markers, as they are stored in the bladder following alcohol intoxication [13].

We clearly show that even a single but high dose of alcohol induces an increase in FUC activity, suggesting ethanol-induced liver damage. It is consistent with earlier observations of increased activity of serum and urinary exoglycosidase (β-hexosaminidase) after an episode of acute alcohol intoxication [8]. As we demonstrated good sensitivity and specificity of urinary FUC and a strong positive correlation between serum FUC and AST and a strong inverse correlation between urinary FUC and serum ALT after a binge drinking session, we may consider urinary FUC activity as an indicator of liver damage. Aspartate aminotransferase is generally a better and more specific marker of alcohol-induced liver damage than ALT [13]. In our study, activity of ALT did not change and AST decreased on the 5th day after intoxication, while urinary FUC was raised on the 5th day. Urinary FUC seems to be a long lasting laboratory marker of excessive drinking (binge drinking), but confirmation of these results requires further research based on a relatively large sample to be sufficiently representative of a vast population.

Conclusions

Even a single but large dose of alcohol is capable of causing an increase in urinary activity of α-L-fucosidase, which is associated with liver damage during alcohol ingestion, and can be detectable five days thereafter.

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Experimental & Clinical
Hepatology

Received: 2012.12.05
Accepted: 2012.12.10
Published: 2012.12.20

Is matrix metalloproteinase-9 (MMP-9) a new prognostic marker of liver inflammation?

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: Self financing

Summary

MMP-9 is a zinc-dependent metaloproteinase involved in many physiological and pathological processes (morphogenesis, liver inflammation). MMP-9 prompted many investigators in hope that it will become a new prognostic marker of inflammation and fibrosis. Studies show a positive correlation between MMP-9 concentration and inflammation but not fibrosis. MMP-9 may play a role in the diagnostic process as a non-specific inflammatory marker.

Key words: **MMP9 • hepatitis • HBV • HCV • cancer • sclerosis • non-small-cell lung cancer • asthma • COPD • sepsis**

Full-text PDF: http://www.expclinhep.com/fulltxt.php?ICID=883664

Word count: 1720
Tables: –
Figures: –
References: 23

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Background

The MMP (matrix metalloproteinase enzymes) family plays a central role in the breakdown of extracellular matrix in in biological processes, for instance polyneurophaty, where higher level of MMP-9 in alcoholic polyneurophaty reached the level of statistic relevance [1]. The function of MMPs is to degrade all kinds of extracellular matrix proteins. They can also modify bioactive molecules. MMPs are involved in cleavage of cell surface receptors, release of apoptotic ligands (FAS ligand or CD95L – a type-II transmembrane protein, which belongs to the tumor necrosis factor (TNF) family), chemokine and cytokine inactivation or activation [2].

Matrix metalloproteinase proteins, also called matrixins, are zinc-dependent. They are distinguished by their dependence on substrates. Collagen is a substrate for MMP-1, -8, gelatine for MMP-2, -9, stromelysin for MMP-3, -10, -11, matrilisyn for MMP-7, membrane for MMP- 14, -15, -16, -24 and metaloelestase for MMP-12 [1].

Deregulation of MMPs was implicated in many diseases including: hepatitis, arthritis, metastasis, chronic ulcers, encephalomyelitis and cancer. MMP activity is regulated by two major endogenous inhibitors: tissue inhibitors of metalloproteases (TIMPs) and alpha2-macroglobulin. Most MMPs are secreted as inactive pro-proteins, which are activated when cleaved by extracellular proteinases [3]. The enzyme encoded by this gene is involved in cleavage of type I and V gelatine and type IV and V collagen [4].

MMP-9 as a Subject of Study of Many Medical Domains

The role of MMP-9 in pathology is focused on specific diseases. Sepsis is a medical condition characterised by an inflammatory state of the whole body [5]. For that reason, MMP levels are the subject of this study. Some results indicate that MMPs and TIMPs may play a crucial role in severe sepsis. Other results show significantly higher levels of MMP-9, MMP-8 and TIMP-1 in severe sepsis compared to healthy controls [6].

In asthma and COPD, chronic inflammatory diseases of the airways, levels of MMP-9 were significantly higher than in healthy controls. Serum concentrations of MMP-9 correlate with the stage of COPD. That proves a role of MMP-9 in inflammation [7–9].

Type IV collagenases, MMP-9 and MMP-2 are involved in degradation of vascular membrane bases, contributing to cancer metastasis.

Kopczyńska E. et al demonstrates that MMP-9 and MMP-2 concentrations in serum did not differ significantly between groups with various T- and N-factors. Serum MMP-9 level was significantly higher in patients with metastases than in those without them. Patients with inoperable tumours (III B–IV) had significantly higher serum MMP-9 concentrations compared to those with resected tumours (I–IIIA). The final conclusion is such that serum MMP-9 may be a marker of metastasis in non-small-cell lung cancer [10].

Other study by Dziankowska-Bartkowiak B. shows lower levels of MMP-9 in the sera of patients with skin diseases compared to the control group [11]. Systemic sclerosis (SSc) is an autoimmune disease that can potentially involve all tissues and organs of the human body.

Another study showed decreased levels of MMP-9 in a group of patients with pulmonary arterial hypertension in the course of systemic sclerosis [12].

Hypertension is a major cause of cardiovascular remodelling. In the cardiovascular system, remodelling of the extracellular matrix is controlled by matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs (TIMPs). Results of the study suggest that MMP-2, MMP-9 and TIMP-1 may play a role as biomarkers of cardiovascular remodelling in hypertension. If these results are confirmed in prospective clinical studies, they could provide new tools to stratify cardiovascular risk in hypertensive patients [13].

Liver Diseases

Reports regarding MMP-9 are very interesting but controversial and a lot of attention was devoted to those markers lately. In order to avoid the need of liver biopsy, many investigators focused on MMP-9 as a marker of inflammation and fibrosis in chronic hepatitis C. In the study, 8 non-hepatitis C patients with normal concentrations of liver enzymes and 35 patients with chronic hepatitis C underwent liver biopsy. The severity of inflammation and stage of fibrosis were determined by the Desmet score on a scale of 0–4. Patient and control groups were similar in terms of age and male/female ratio. MMP-9 activity in the serum and liver tissue was elevated in patients compared to controls ($p<0.05$). The highest rise in serum MMP-9 level was observed in grades 2 through 3 and was greater than the increase in serum transaminase levels, indicating its advantage in assessing the progression of disease activity. Serum MMP-9 values correlated with liver inflammatory grade in histological examination ($p<0.05$) but not with the stage of fibrosis [14].

Serum MMP-9 is decreasing in the course of progression of chronic hepatitis to cirrhosis, with the lowest levels observed in the cirrhotic group [15]. No correlation between liver MMP activity and liver fibrosis or inflammation was observed [14].

Another study examined the possible association of mmp9 gene polymorphisms with progression of chronic liver disease in the Japanese population. Researchers tested 91 patients with HCV-related chronic hepatitis and 89 patients with HCV-related liver cirrhosis. They determined MMP-9 C/T polymorphism and in MMP-9 genotypes, the C homozygotes and C allele frequencies were significantly greater in the group with liver cirrhosis than in the chronic hepatitis group [16]. The degree of liver fibrosis and inflammation is important in patients with chronic hepatitis C (CHC) in terms of therapy as well as prognosis. Therefore, serum MMP-9 can serve as a marker of disease activity [14]. Study evaluated involvement of MMP-9 in relation to viral load in the development of liver dysfunction in HBV and HCV. Blood samples from 20 patients chronically infected with HBV and 30 with HCV, along with 15 healthy individuals as controls, were investigated. The following results

were obtained in this study: levels of MMP-9 were significantly higher in HCV than in HBV patients ($p<0.01$) and positively correlated with HBV viral load ($p<0.01$) as well as AST: ALT ratio ($p<0.05$). Therefore, MMP-9 levels could reflect progressive liver damage in HBV and HCV infection. However, there is a suggested difference between pathological HCV and HBV mechanism, as HCV probably promotes hepatocyte damage and fibrosis through mechanisms other than replication. Continuous expression of HBV genome through replication and secretion of viral antigens may contribute to transcriptional regulation of MMP-9, consequently promoting liver damage and fibrosis [17]. Another study shows an altered pattern of circulating matrix metalloproteinases -2,-9 and tissue inhibitor of metalloproteinase-2 in patients with HCV-related chronic hepatitis. This study confirms an altered pattern of metalloproteinases and their tissue inhibitors and such alterations can contribute to development of liver fibrosis [18].

Another study evaluated serum matrix metalloproteinase MMP-9 to MMP-2 ratio as a biomarker in hepatocellular carcinoma. Serum samples from 181 chronic hepatitis B patients (52 healthy carriers, 47 chronic hepatitis patients, 50 cirrhosis patients, and 32 HCC patients) were collected. Results were analyzed using the Receiver Operating Characteristic (ROC) curve. Test result showed that serum MMP-9/ MMP-2 ratios in HCC patients were significantly higher than those in healthy carriers, chronic hepatitis patients, and cirrhosis patients ($p=0.004$, 0.034, and< 0.001, respectively). Sensitivity and specificity at the optimal cut-off point equal to 0.97 were 69.7% and 73.4%, respectively. Significantly higher MMP-9/MMP-2 ratios were found in advanced, inoperable HCC patients compared to those at an early stage of HCC ($p=0.005$).

No significant differences were found for alpha-fetoprotein levels between these two groups ($p=0.312$), thus serum MMP-9/ MMP-2 ratio can be used as an accessory diagnostic marker in hepatitis B virus-related HCC. This ratio is useful in distinguishing between patients at an early stage of HCC and those with advanced HCC [19].

The relationship between HCC and MMP-9 is still an interesting issue for scientists. They took tetraspanin CD 151 into consideration. Tetraspanin CD 151 is involved in several pathological activities associated with tumour progression, including neoangiogenesis.

During their research, they found a positive correlation between the levels of MMP-9 and CD 151 expression in HCC cells. Overexpression of CD 151 facilitated expression of MMP-9. In contrast, down-regulation of CD 151 expression diminished the ability of HCC cells to form microvessels *in vitro* and reduced their *in vivo* metastatic potential. It shows that CD 151 may be useful as a high-priority therapeutic target against angiogenesis in HCC [20].

A different study presents the role of MMP-9 and MMP-2 in HCC. Research compared clinical efficacy of MMP-9 and MMP-2 overexpression in predicting tumour recurrence and survival after surgical resection in HCC patients. The conclusion from this article was that MMP-9 is superior to MMP-2 with regard to prediction of tumour recurrence and survival in HCC patients after surgical resection [21].

Lipocalin-2 (LCN2) forms complexes with MMP-9 and can be detected in the urine of patients with several types of cancers. The goal of the study was to examine the relationship between urinary LCN2 levels and MMP-9 activity with respect to the stage of liver fibrosis in patients with chronic hepatitis C (CHC) and to assess the utility of urinary LCN2 as a non-invasive marker of hepatic fibrosis [22].

Patients with HIV infection exhibited a striking increase in TIMP-1 levels, which is more evident in patients with advanced CD4 depletion. There was no elevation in plasma concentrations of MMP-9. The highest levels of TIMP-1 were found in the HIV/HCV co-infected patients. The values of TIMP-1 in HIV-infected patients with chronic HCV hepatitis were significantly higher than in HIV-positive individuals without HCV infection, even including those with low CD4 count. No significant differences were seen in the MMP-9 levels [23].

Conclusions

MMP-9, as an enzymatic protein involved in the process of tissue remodelling, is a potential diagnostic marker. Many medical trials are analyzing correlations between the level of serum MMP-9 and degree of fibrosis or inflammation. The results of many research studies make us believe that MMP-9 is going to be an important marker in the future. According to the research, level of serum MMP-9 is increased in inflammatory diseases such as COPD, asthma or sepsis. This enzyme may be useful for assessment of metastases of non-small-cell lung cancer or stratifying cardiovascular risk in patients with hypertension.

Studies on MMP-9 in liver diseases show that it may play an important role in the diagnosis of HCV, HBV and HCC. Serum MMP-9 has an advantage over liver transaminases in assessment of disease progression. However, serum MMP-9 values correlated with the histological grade of liver inflammation but not the stage of fibrosis. MMP-9/MMP-2 ratio may play a role in grading of HCC and predicting recurrence and survival after surgical resection.

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Experimental & Clinical
Hepatology

Received: 2012.11.26
Accepted: 2012.11.30
Published: 2012.12.21

Hyaluronic acid and tissue inhibitor of metalloproteinase 1 predict advanced liver fibrosis in children with autoimmune hepatitis – preliminary report

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Source of support: This study was supported by a grant from the Medical University of Bialystok, Poland No 3-43665L

This study was presented during 44th Annual Meeting of ESPGHAN (25–28 May, 2011, Sorrento, Italy)

Summary

Background: Liver biopsy is a gold standard for the assessment of liver fibrosis. The biopsy is an invasive procedure and alternative methods of assessment of liver fibrosis are under investigation. Therefore, we studied four potential serum markers of fibrosis and compared with histopathological findings in liver biopsy in children with autoimmune hepatitis (AIH).

Material/Methods: Fasting serum levels of hyaluronic acid (HA), laminin, MMP-9 and TIMP-1 were measured in 29 children with biopsy-verified AIH. Diagnoses of viral hepatitis (HBV, HCV) and metabolic liver diseases (Wilson's disease, alpha-1-antitripsin deficiency) were excluded. Batts and Ludwig scoring system was used to determine the staging of liver disease. Receiver operating characteristics analysis was used to calculate the power of the assays to detect advanced liver fibrosis.

Results: Mild liver fibrosis (stage 1–2) was present in 23 and advanced fibrosis (staging 3–4) in 6 patients. Children with advanced fibrosis had significantly higher HA (p=0.02) and TIMP-1 (p=0.03) levels than children with mild fibrosis. Significant positive correlation was found between disease staging and HA or TIMP-1 concentrations. Significant predictive ability to differentiate children with advanced fibrosis from those with mild fibrosis was found for HA (AUC=0.7681, p=0.02) and TIMP-1 (AUC=0.7935, p=0.015). HA concentration >85.1 ng/ml had a sensitivity of 83% and a specificity of 87% and TIMP-1 level >282 ng/ml had a sensitivity of 83% and specificity of 74%. Laminin and MMP-9 did not allow for a useful prediction.

Conclusions: HA and TIMP-1 levels differentiated children with advanced fibrosis from those with mild fibrosis and thus these non-invasive parameters can be useful in following progression of AIH in children.

Key words: **autoimmune hepatitis • children • liver fibrosis • hyaluronic acid • laminin • MMP • TIMP**

Full-text PDF: http://www.expclinhep.com/fulltxt.php?ICID=883687

Word count: 1817
Tables: 2
Figures: 1
References: 27

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Background

Autoimmune hepatitis (AIH) is characterized by inflammatory infiltration and fibrosis of liver tissue, presence of non-specific circulating autoantibodies and increased level of immunoglobulin G in the absence of a known etiology [1]. AIH may present with an insidious or acute onset. The course of the disease in children is usually more aggressive than in adults [2,3].

AIH diagnostics include blood tests and liver biopsy [4,5]. Liver histology is necessary to describe the activity of the inflammatory infiltrate and the stage of liver fibrosis. This information is necessary for diagnosis and, furthermore, it allows tracking therapy-related changes in disease activity and staging. At the moment, liver histology is a gold standard for the assessment of liver fibrosis. However, liver biopsy is an invasive and painful procedure and there is a risk of important complication and thus, alternative methods of assessment of liver fibrosis are under investigation [6–9].

There are many extracellular matrix (ECM) components that can be potentially used to monitor connective tissue development. In this study we chose hyaluronic acid (HA), laminin, matrix metalloproteinase-9 (MMP-9) and tissue inhibitor of matrix metalloproteinase-1 (TIMP-1). In the previous studies conducted by Lebensztejn et al. [10,11] the usefulness of hyaluronic acid, laminin and MMP-9 in predicting liver fibrosis in children with chronic hepatitis B was confirmed. Moreover, during progression of chronic liver disease (e.g. AIH) an imbalance occurs between synthesis and breakdown of ECM components. MMPs are involved in degrading ECM while TIMPs prevent their fibrolytic action [12].

To our knowledge, serum markers of fibrosis have not yet been studied as predictors of liver fibrosis in children with AIH. Therefore the aim of the study was to evaluate whether measurements of HA, laminin, MMP-9 and TIMP-1 concentrations may have clinical application as markers of liver fibrosis in this group of patients.

Material and Methods

Material

The study was carried out prospectively on 29 unselected, consecutive children (mean age 14.5 years, range 5.5–17.8, 8 boys and 21 girls) with AIH, who were admitted to Department of Gastroenterology, Hepatology and Immunology of Children's Memorial Health Institute, Warsaw, Poland between Aug 2009 and Aug 2010. The diagnosis of AIH was based on liver biopsy, increased ALT, immunoglobulin G, gammaglobulin levels and presence of autoantibodies. All children had type I AIH. The disease was diagnosed at the age of 3.6–17.6 years (11.8±3.7 years). Other causes of chronic liver disease, such as HBV or HCV co-infection and metabolic liver disorders (Wilson's disease, alpha-1-antitripsin deficiency) were excluded. Children with the evidence of other acute or chronic infections were excluded from this study. Seven children had the first flare of AIH and 22 were admitted for routine control of AIH. All children received standard therapy with prednisone and/or azathioprine and treatment duration varied from 0 to 6 years (2.7±2.1 years).

Table 1. Biochemical and histological characteristic of study population.

Patient parameters	Mean	SD
Age (years)	14.5	3.8
ALT (IU/l)	90	154
Immunoglobulin G (mg/dl)	1670	720
Gammaglobulins (g/l)	16.4	7.8
Stage	1.87	0,9
Grade	1.48	1,4
HA (ng/ml)	70.6	159.8
Laminin (ng/ml)	653	494
TIMP-1 (ng/ml)	610	126
MMP-9 (ng/ml)	478	345

HA – hyaluronic acid; MMP – matrix metalloproteinase; TIMP – tissue inhibitor of matrix metalloproteinase.

Twenty-three children (mean age 11.7±4.5 years) without anamnestic, clinical or laboratory signs of liver diseases or other chronic diseases were included as control group.

Informed consent was obtained from all of patients' parents and the protocol was approved by the ethics committee of the Medical University of Bialystok, Poland.

Standard biochemical tests were performed directly by validated automated methods and included total bilirubin, alanine aminotransferase (ALT), gammaglobulin, and immunoglobulin G (IgG) concentrations.

Methods

Measurement of serum markers of fibrosis

Hyaluronic acid, laminin, MMP-9 and TIMP-1 levels in serum samples were assessed with ELISA technique (obtained after an overnight fast) using a commercial kit (HA: Corgenix, laminin: Takara, MMP-9 and TIMP-1: R&D Systems).

Histological analysis

All children underwent liver biopsy on the day following serum sampling. Liver specimens were fixed in buffered formalin and embedded in paraffin. Histological sections were stained with hematoxylin-eosin and AZAN. Stage of fibrosis and inflammation grade were assessed in a blinded fashion by a single pathologist without knowledge of the patients' laboratory or clinical data. In order to determine the specificity and sensitivity of the assay we arbitrarily defined advanced liver fibrosis as a score >2 according to Batts and Ludwig classification [13].

Statistical analysis

Serum concentrations of biochemical markers were expressed as mean values ± standard deviations (SD). Statistical analysis was performed with the Mann-Whitney two-sample

Table 2. Comparative characteristics of children with AIH with mild (stage 1–2) and advanced (stage 3–4) fibrosis.

Patient parameters	AIH children with mild fibrosis (n=23) Mean±SD	AIH children with advanced fibrosis (n=6) Mean±SD	P
Age (years)	14.1±4.0	16.3±1.9	NS
Sex (F/M)	17/6	4/2	NS
ALT (IU/l)	80±167	128±92	0.04
ImmunoglobulinG (mg/dl)	15.7±5.8	18.2±6.0	NS
Gammaglobulins (g/l)	15.7±8.1	18.4±3.6	NS
Grade	1.0±1.1	3.2±0.4	0.0012
HA (ng/ml)	53.0±168.1	138.4±108.3	0.02
TIMP-1 (ng/ml)	254±128	352±88	0.03
Laminin (ng/ml)	722±397	635±523	NS
MMP-9 (ng/ml)	305±208	523±363	NS

HA – hyaluronic acid; MMP – matrix metalloproteinase; TIMP – tissue inhibitor of matrix metalloproteinase.

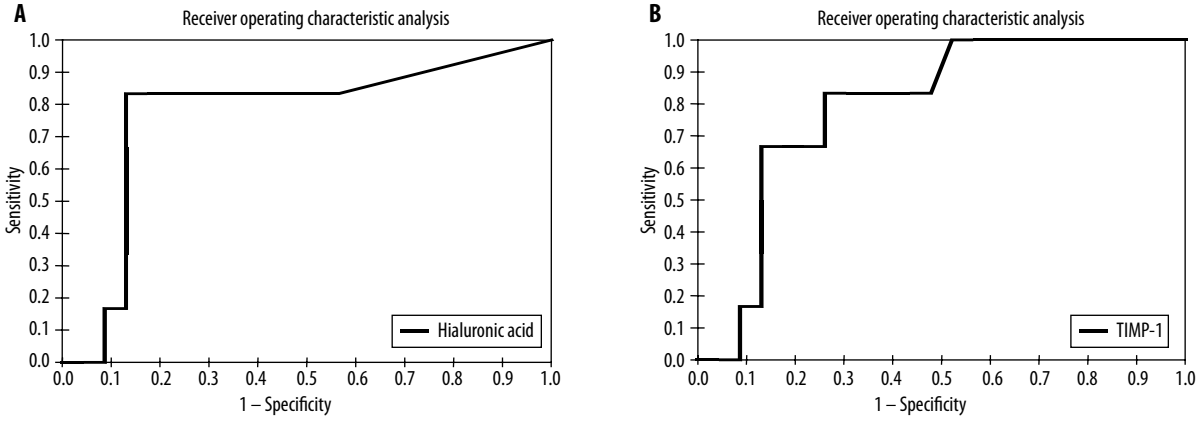

Figure 1. ROC curve of predictive ability of serum HA (**A**) and TIMP-1 (**B**) in detecting advanced liver fibrosis in children with autoimmune hepatitis according to the Batts and Ludwig scheme. AIH – autoimmune hepatitis; AUC – area under curve; F – fibrosis; HA – hyaluronic acid, MMP – matrix metalloproteinase; TIMP – tissue inhibitor of matrix metalloproteinase; ROC – receiver operating characteristics.

test for nonparametric data. The relationship between biochemical tests and liver histology scores was analyzed by the Spearman rank-correlation test. Tests were considered statistically significant at $p<0.05$. Receiver operating characteristics (ROC) analysis (AccuROC, Montreal, Canada) was used to calculate the power of the assay to detect advanced liver fibrosis. Sensitivity of the assay was plotted against the false positivity (1-specificity). Comparison of the area under curve (AUC) was performed using a two-tailed p-test, which compares the AUC to the diagonal line of no discrimination (AUC 0.5).

Results

Characteristics of the patients

Selected biochemical and histological data are presented in Table 1. On the day of the study, 14 (48%) of patients had abnormal ALT activity, 10 (34%) had increased IgG concentrations and 8 (28%) presented with elevated gammaglobulin levels. Liver biopsy showed mild to moderate disease activity (grade 0–2) in 21 (72%) and high activity (grade 3–4) in 8 (28%). Mild fibrosis (stage 1–2) was present in 23 (79%), while advanced fibrosis (stage 3–4) was present in 6 (21%) subjects. Patients with advanced fibrosis had higher ALT values (128±92 *vs.* 80±167 U/l, p=0.04) and grading (3.2±0.4 *vs.* 1.0±1.1, p=0.0012) than those with mild fibrosis (Table 2).

Serum concentrations of markers of fibrosis

Serum concentration of HA was significantly higher (p=0.0004) in AIH patients (70.6±159.8 ng/ml) compared to controls (0.9±2.6 ng/ml). TIMP-1 level was also higher (on the verge of reaching statistical significance) (p=0.08) in children with AIH (274±126 ng/ml) than in control group (208±49.5 ng/ml). Laminin and MMP-9 level was higher in children with AIH than in controls but it did not reach statistical significance (p>0.05).

Patients with advanced liver fibrosis identified in histological examination had higher concentrations of HA (138±108

vs. 53±168 ng/ml, p=0.02) and TIMP-1 (352±88 *vs.* 254±128 ng/ml, p=0.03) than those with mild fibrosis. No differences in laminin and MMP-9 levels between patients with various stages of fibrosis were observed (Table 2).

Diagnostic value of serum fibrosis markers for identification of patients with advanced liver fibrosis

Significant positive correlation was found between disease staging and HA (r=0.53, p=0.0028) as well as staging and TIMP-1 (r=0.42, p=0.024), while no correlation between laminin or MMP-9 and staging was observed. HA (AUC=0.7681, p=0.02) and TIMP-1 (AUC=0.7935, p=0.015) levels were associated with significant predictive value in differentiating children with advanced fibrosis from those with mild fibrosis (Figure 1A, 1B). HA level >85.1ng/ml had a sensitivity of 83% and a specificity of 87%, while TIMP-1 >282 ng/ml had a sensitivity of 83% and specificity of 74%. Laminin and MMP-9 did not allow a useful prediction.

Discussion

AIH is a serious disease and leads to liver failure and death if left untreated. Effective treatment can control inflammation and slow down progression of the disease, thus the prognosis is much better in patients receiving therapy than in the untreated and neglected cases. The current standard of care in AIH includes a combination of prednisone and azathioprine in long-term treatment. Induction treatment starts with high doses of prednisone administered for 4–6 weeks, which are later tapered to low doses in remission maintenance therapy. The doses of prednisone and azathioprine during maintenance treatment are adjusted according to clinical efficacy measured by liver function tests and therapeutic safety measured by the presence of side effects. Thus patients with AIH must be under permanent clinical observation by an experienced physician. Laboratory tests are performed on outpatient basis every 3–6 months and routine liver biopsy is repeated every 2 years. Result of control liver histology is the major issue in the decision-making process concerning further therapy. Patients without inflammatory infiltrates (grading = 0) can attempt to discontinue the therapy while patients with at least minimal inflammatory infiltrations should continue treatment, as the risk of hepatitis exacerbation is high after stopping treatment [14–16]. However, the role of non-invasive surrogate markers in grading the liver inflammatory infiltration (such as ALT and IgG) seems to be clearly defined [17,18], but the data concerning serum fibrosis markers are lacking.

The group of patients described in this paper represents typical pediatric AIH. It consisted of patients with newly diagnosed disease and patients who came for routine disease grading and staging assessment. Almost half of patients (48%) had abnormal liver function tests. Very similar results (abnormal ALT in 45% of subjects) were obtained in the AIH survey conducted in Poland in 2009 [19]. In this group of patients 21% had marked liver fibrosis. This figure is slightly lower than that reported for children with AIH by Mieli,-Vergani & Vergani [3]. This may be due to aggressive therapy administered to our patients.

In this study, for the first time we found significantly higher levels of HA and TIMP-1 in AIH children with advanced liver fibrosis compared to patients with mild fibrosis. We also demonstrated significant correlation of these biomarkers with the stage of liver fibrosis. Using ROC analysis we confirmed that it is possible to differentiate between children with advanced fibrosis and those with mild liver fibrosis using HA and TIMP-1. To our best knowledge it is the first study analyzing serum levels of fibrosis markers in a homogenous group of patients with AIH. Only Parsian et al. [20] evaluated HA and laminin concentrations in adult patients with various etiologies of chronic hepatitis (mainly HBV and HCV infections) and only 13 of them had AIH. They found that HA could be used as a noninvasive biomarker to discriminate between patients with mild and severe fibrosis, but contrary to our study, they also confirmed the predictive significance of laminin. The findings concerning HA are also in agreement with our previous studies in children with biopsy-proven chronic hepatitis B [11] and non-alcoholic fatty liver disease, [21] in which we confirmed the potential of this marker for predicting liver fibrosis. Many authors explored HA as a promising biomarker for prediction of liver fibrosis as well [22,23], but findings concerning TIMP-1 are conflicting. Leroy et al. [24] showed association between the stage of liver fibrosis and TIMP-1 level, but Macias et al. [25] did not. In children, clinical usefulness of this marker for prediction of liver fibrosis was confirmed in HCV patients by Valva et al. [26] and in HBV patients by Lebensztejn et al. [11].

Present study has some limitations, such as a rather limited number of participants. Therefore, a small sample size makes it difficult to validate the utility of examined biomarkers. Moreover we also did not analyze the length of liver biopsy specimen. For that reason, a sampling error is possible [27].

Conclusions

HA and TIMP-1 levels differentiated between children with advanced fibrosis and those with mild fibrosis and therefore, these non-invasive parameters may be useful in identification of patients at risk of disease progression and in following progression of AIH in children. However, this finding needs to be confirmed in larger studies.

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Experimental & Clinical Hepatology

Received: 2012.02.05
Accepted: 2012.02.27
Published: 2012.06.01

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

Methecillin Resistant *Staphylococcal aureus* (MRSA) isolates from a hepatology intensive care unit in Egypt: A silent killer

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Summary

Background: Hospital-acquired infections due to MRSA are associated with considerable morbidity, mortality, and excess costs. Our work aimed to study the prevalence, risk factors and genotypic features of MRSA isolated from the hepatology ICU inpatients of Mansoura Specialized Medical Hospital (MSMH) during the study period.

Material/Methods: A total 184 of *Staphylococcus aureus* clinical samples were collected between January 2009 to September 2011. Isolated colonies were identified in a systematic manner for selection of MRSA.Oxacillin and cefoxitin resistance tests identified MRSA that were subjected to antibiogram and resistogram. MRSA amplified genes, visualized by agarose gel electrophoresis, were analyzed by Sanger sequencing.

Results: Only 49 isolates out of the *Staphylococcus aureus* isolated strains were identified as MRSA strains by cefoxitin disc diffusion test. All strains are resistant to cefoxitin, ceftriaxon, cloxacillin and ampicillin while most strains were susceptible to vancomycin. Sanger sequencing of *mecA* gene showed 3 polymorphs. MRSA isolates were more form blood samples ($P<0.05$), taken from male patients, above 60 years ($P<0.01$), of low socioeconomic status ($P<0.01$), of >2weeks duration hospital stay ($P<0.01$), with a previous history of hospitalization within the past year ($P<0.01$), suffering from chronic disease ($P<0.001$), and a positive history of antibiotic use in the last 6 months ($P<0.001$). Positive family history of chronic disease ($P<0.001$), hospitalization within the last year ($P<0.01$) and the presence of a family member working in a clinic or a hospital ($P<0.05$) were noted in MRSA-positive patients.

Conclusions: Our data revealed an increased prevalence of multi-drug resistant MRSA isolates where PCR was of the best choice for their rapid and accurate detection.

An effective infection control program should be implemented for appropriate MRSA management.

Key words: **methicillin • prevalence • resistance • *Staphylococcus aureus***

Full-text PDF: http://www.expclinhep.com/fulltxt.php?ICID=997404

Word count: 2198
Tables: 7
Figures: 2
References: 31

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Background

MRSA is an increasingly common complication in health care and has become endemic worldwide within the past two decades [1]. The spectrum of MRSA infection varies from mild skin infection to serious and invasive disease such as septicaemia, endocarditis and deep-seated abscesses [2].

The progressive emergence and rapid dissemination of antibiotic resistance in *Staphylococcus aureus* constitute a major health concern and have been considered as a global crisis [3].

MRSA prevalence varies markedly between different countries and between different regions and hospitals [4]. Colonization rates as high as 6% have been reported among staff at institutions where MRSA is a problem. Staff members with current sinus or respiratory infections or active dermatitis are at increased risk of transmitting MRSA if they have become colonized [5]. Our work aimed to study the prevalence, risk factors and genotypic features of MRSA isolated from the hepatology ICU inpatients of MSMH during the study period.

Material and Methods

A total 184 *Staphylococcus aureus* specimens were collected from urine, sputum, wounds and blood from the hepatology ICU inpatients of MSMH between January 2009 to September 2011. Specimens were collected either by sterile swabs (wounds) or in sterile containers (blood, sputum, vaginal discharge and urine). All regulations concerning specimen collection were thoroughly followed for a proper sampling technique [6].

Representative samples from the collected specimens were propagated into nutrient broth tubes and incubated at 37°C for 24 hrs, then, inoculated on plate of mannitol salt agar (MSA) as a specific media for SA and incubated at 37°C for 48 hrs to observe fermentation of MSA as yellow color. After incubation, separate colonies were picked up. Pure culture was prepared on appropriate media for further identification.

Colonies of *Staphylococcus aureus* isolates were picked up and identified in a systematic manner for MRSA selection. First, the observation of MSA fermentation [7], then isolates were tested for morphology of their colonies and Gram stain positivity, production of catalase for differentiation from *Streptococci* [8], production of coagulase for differentiation of *Staphylococcus aureus* from other *Staphylococcal* species [9], Deoxyribonuclease activity [10], finally oxacillin [11] and cefoxitin [12] resistance tests for isolates to identify MRSA.

The selected isolates that fulfilled MRSA diagnostic criteria were inoculated on nutrient agar slopes. After an overnight incubation at 37°C, the slopes were stored at 4°C. Passage of the isolates was done every 4–5 weeks. Also, before starting any experiment, subculture was done twice to allow cells to restore their viability [13].

Antibiotic susceptibility testing by disc diffusion method [14]: Isolates were reported as sensitive or intermediate or resistant from the respective interpretation charts [15].

The minimal inhibitory concentrations (MICs) of ciprofloxacin, gentamicin, flocloxacillin and ceftriaxon against the isolated strains of MRSA were determined by agar dilution method. The MIC was defined as the lowest concentration of the antimicrobial agent that inhibited the tested isolate growth. Negative and positive controls were simultaneously carried out to ascertain both sterility and growth promoting ability of the medium [16].

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) analysis

SDS-PAGE of total cell protein extracts of the tested MRSA strains produce patterns containing 6-12 discrete bands with molecular weights in the range (14.4 to 116 KDa). Similar SDS-PAGE patterns were considered as one pattern and photographed [17].

Sequencing of the *mecA* gene

The amplified genes were visualized by agarose gel electrophoresis and 1% ethidium bromide staining under U.V. trans-illuminator. The PCR products of the whole genes were sequenced according to the applied bio-system big dye terminator v3.1 DNA sequencing reaction in a 96 well format protocol [18].

Statistical analysis

Student's *t*-test and Chi-square test were used to assess differences among the studied groups. Multivariate logistic analysis was used to evaluate the influence of variables on prevalence. $P<0.05$ was considered to be statistically significant. Statistical analyses were made using SPSS 17.0 (SPSS, Chicago, IL, USA)

Results

Of 1007 over all collected specimens during the study period, a total 184 of *Staphylococcus aureus* clinical specimens were identified as Gram-positive cocci, non-motile, arranged in irregular grape-like clusters based microscopic examination. Morphologically, colonies were rounded, smooth, raised and glistening on nutrient agar. On MSA, these colonies caused fermentation where red color turned into yellow and gave positive results for catalase and coagulase tests.

Table 1 showed that MRSA isolates were more significant from blood samples ($P<0.05$), taken from male patients, above 60 years ($P<0.01$), of low socioeconomic status ($P<0.01$), of >2weeks' duration of hospital stay ($P<0.01$), with a previous history of hospitalization within the past year ($P<0.01$), suffering from chronic disease ($P<0.001$), and a positive history of antibiotic use in the last 6 months ($P<0.001$). Positive family history of chronic disease ($P<0.001$), or hospitalization within the last year ($P<0.01$) and the presence of family member working in a clinic or a hospital ($P<0.05$) were noted in MRSA-positive patients.

All studied MRSA-positive ICU patients (n=49) were suffering from hepatic encephalopathy (HE) precipitated by gut bleeding (n=19), chronic active hepatitis (n=11), diarrhoea (n=13), and septicaemia (n=6). All vancomycin-susceptible patients (45/49) decolonized [(45/45) (100%)] after full

Table 1. Clinical and demographic characteristics of the participants.

Parameter	Colonization status			
	Total participants per variable group	Non colonized	*S. aureus*	MRSA
Total (n.)	1067	885	182	49
Age (years) mean ±SD	56.3±2.1	55.4±1.7	57.3 ± 3.9	61.7±4.1**
Gender (M:F)	553:514	456: 429	97: 85	36: 13**
Socioeconomic status:				
Low	343	287	56	36**
Average	519	410	109	8
High	205	188	17	5
Sample(site) distribution:				
Wound	42	27	15	2
Blood	426	404	22	14*
Urine	224	181	43	14
Vaginal discharge	73	46	27	5
Sputum	215	173	42	4
Skin infection	87	54	33	10
Personal risk factors:				
History of antibiotic use in the last 6 mo.	77	38	39	33***
Hospitalization within the last year	82	44	38	27**
Duration of hospital admission:				
<1wk	41	28	13	4
1–2 wks	37	24	13	5
>2 wks	41	16	25	18**
Presence of chronic disease	85	36	49	39***
Household risk factors:				
Household pets	14	9	5	1
Family member with chronic disease	73	29	44	38***
Family member work in a clinic or hospital	60	33	27	13*
Family member hospitalization within 1 yr	79	31	48	21**

* P-value <0.05; ** *P*-value <0.01; *** *P*-value <0.001.

dose parenteral therapy with an over all recovery of [35/49 (71.42%)] of all the participants as shown in Table 2.

Only 49 isolates out of the isolated strains of *Staphylococcus aureus* (184) were identified as MRSA isolates that gave a positive DNase test and exhibited resistance by oxacillin and cefoxitin agar screening tests and grew at 37°C and optimal temperature for pigment formation was 20–25°C (Table 3).

Table 3 showed the result of oxacillin disc diffusion test where 43 samples were MRSA, and the remaining 6 isolates were reported as MSSA when these interpretative ranges were used [15]; resistant (R) ⩽10 mm; Intermediate (I) 11–12 mm; S ⩾13 mm. New interpretative ranges [12] were used to distinguish MRSA from MSSA. Those with a zone diameter of ⩽19 mm were scored as resistant and those with a zone diameter of ⩾20 mm were reported as susceptible. Accordingly, all tested isolates (n=49) were considered as MRSA by cefoxitin disc diffusion test.

Table 4 showed fourteen different antibiotic susceptibility patterns of all tested MRSA isolates using disc diffusion

Table 2. Vancomycin susceptibility in MRSA-positive participants.

HE pricipitants (n., %)	Vancomycin susceptibility and subsequent MRSA decolonization (n.,%)		Recovery after Vancomycin therapy (n.,%)	
Gut bleeding [19, (38.77%)]	18	(36.73%)	11	(22.44%)
Chronic active hepatitis [11, (22.44%)]	10	(20.40%)	9	(18.36%)
Diarrhoea [13, (26.35%)]	12	(24.48%)	12	(24.48%)
Septicaemia [6, (12.24%)]	4	(8.16%)	3	(6.12%)

Table 3. Oxacillin and cefoxitin susceptibility tests of MRSA isolates.

No. of isolates	Inhibition Zone diameter of Oxacillin (mm)	Inhibition Zone diameter of Cefoxitin (mm)
1	0	6.3
2	0	6.5
3	0	0
4	0	6.5
5	0	0
6	6.3	0
7	7.4	0
8	7.3	0
9	0	0
10	0	0
11	0	0
12	0	0
13	6.2	0
14	0	0
15	8	8
16	7.3	7.2
17	8	8
18	7.9	0
19	7.3	7
20	8	0
21	0	0
22	10	0
23	14.7	0
24	8	0
25	11.3	0
26	12.7	0
27	0	0
28	9.6	0
29	14.6	0
30	0	0
31	0	0
32	0	0
33	0	0
34	10	0
36	0	0
37	7.7	6.9
38	0	0
39	0	0
40	11.4	0
41	14.3	7.3
42	0	0
43	9	0
44	0	0
45	0	0
46	0	0
47	0	0
48	6.4	0
49	6.4	0
50	0	0

method (bioanalyse discs). All strains were resistant to cefoxitin, ceftriaxon, cloxacillin and ampicillin. All samples resistant to oxacillin when the inhibition zone was ≥15 according to the used interpretation data [19]. All strains were

Table 4. Antibiotic sensitivity patterns of MRSA isolates.

Strain(s) no.	Antibiotic sensitivity patterns											No. of strains	%
	OX	FOX	CRO	AMP	CX	VA	CN	AK	NOR	CIP	LEV		
1, 2, 4, 6, 13, 20, 22, 23, 25, 26, 29, 37, 38, 40.	R	R	R	R	R	S	S	S	S	S	S	14	28.57
3, 5, 7, 11, 16, 17, 19, 21, 36, 39, 49	R	R	R	R	R	S	R	R	R	R	S	11	22.45
9,10, 12, 27, 30, 32, 33,42, 45, 46, 47,48	R	R	R	R	R	S	R	R	R	R	R	12	24.49
14, 24	R	R	R	R	R	S	R	S	R	R	R	2	4.08
8	R	R	R	R	R	R	R	R	R	R	S	1	2.04
15	R	R	R	R	R	R	S	R	R	R	S	1	2.04
18	R	R	R	R	R	R	R	R	S	S	S	1	2.04
28	R	R	R	R	R	S	R	S	S	S	S	1	2.04
31	R	R	R	R	R	R	R	R	R	R	R	1	2.04
34	R	R	R	R	R	S	R	R	S	S	S	1	2.04
41	R	R	R	R	R	S	S	R	S	S	S	1	2.04
43	R	R	R	R	R	S	S	S	R	R	R	1	2.04
44	R	R	R	R	R	S	R	S	R	R	S	1	2.04
50	R	R	R	R	R	S	S	R	R	R	R	1	2.04

R – resistance; S – sensitive; OX – oxacillin; FOX – cefoxitin; CRO – ceftriaxon; AMP – ampicillin; CX – cloxacillin; VA – vancomycin; CN – gentamycin; AK – amikacin; NOR – norfloxacin; CIP – ciprofloxacin; LEV – levofloxacin.

susceptible to vancomycin except 4 strains No. 8, 15, 18, and 31. The most important resistance pattern that includes 12 strains resistant to all antibiotics except vancomycin.

Table 5 showed the MICs of the tested antibiotics against MRSA isolates as determined by the standard agar dilution method. All MRSA isolates were highly resistant to ceftriaxon as the MICs ranged from 2 µg/ml to 2048 µg/ml. Regarding flocloxacillin, eight isolates (No.3, 19, 21, 24, 31, 39, 47 and 50) were the most resistant to flocloxacillin and twenty one isolates were flocloxacillin-susceptible. For ciprofloxacin, the MICs range from <0.125 µg/ml to 128 µg/ml. Isolate (No. 50) was the most resistant to ciprofloxacin (128 µg/ml). Six isolates (No.14, 24, 27, 30, 46 and 47) showed a high resistance (64 µg /ml) while ciprofloxacin susceptibility was exhibited by eighteen isolates. Regarding gentamicin, three isolates (No. 47, 48 and 49) were the most resistant to gentamicin (2048 µg/ml) whereas nineteen isolates were gentamicin-susceptible.

Table 6 and Figure 1 showed nine different total cell protein patterns. Similar SDS-PAGE patterns were considered as one pattern. Accordingly, the tested strains were successfully classified into different SDS-PAGE patterns. However, due to the high degrees of similarity observed among these banding patterns, SDS-PAGE analyses of whole-cell extracts can not be used to characterize individual MRSA isolates.

Table 7 showed patterns obtained by agarose gel electrophoresis of amplified *mecA* gene of 41 MRSA isolates from their DNA. Samples no. 24, 26, 28, 38, 39, 40, 46 and 47 were not amplified.

Neighbour-joining trees were constructed to analyze nucleotide sequence similarity and determine the relatedness between isolates. Sanger's sequencing of MRSA isolates characterized the *mecA* gene results in 3 polymorphs, according to their similarities (P=0.002), that can be used in MRSA identification by gene typing (Figure 2).

Discussion

Currently, MRSA is the most common pathogen identified in U.S. hospitals and has been reported worldwide [20]. Hospital-acquired infections due to MRSA are associated with considerable morbidity, mortality, and attributable excess costs [21].

Our study showed some risk factors for MRSA positivity including ICU male patients, above 60 years of age, of low socioeconomic status. Long hospital stay, history of previous hospitalization and intake of broad spectrum antibiotics encourage the emergence of inducible multidrug resistant MRSA strains. Additionally, the presence of family members with chronic illness, with a history of

Table 5. Minimal Inhibitory Concentrations (MICs) of ceftriaxon, flocloxacillin, ciprofloxacin and gentamicin against tested MRSA.

Strains no.	Ceftriaxon (µg/ml)	Flocloxacillin (µg/ml)	Ciprofloxacin (µg/ml)	Gentamicin (µg/ml)
1	2	2	0.5	<0.125
2	4	1	0.25	0.25
3	2048	2048	16	128
4	2	2	0.5	<0.125
5	2048	512	16	128
6	16	2	0.25	0.25
7	1024	256	8	128
8	1024	128	8	128
9	2048	1024	16	128
10	1024	512	8	256
11	4	1	<0.125	0.125
12	2048	1024	16	128
13	4	1	0.5	0.25
14	2048	1024	64	128
15	128	512	8	2
16	2048	512	16	64
17	256	64	8	256
18	32	8	0.25	<0.125
19	2048	2048	8	256
20	8	0.5	0.5	64
21	2048	2048	16	256
22	4	0.25	0.5	0.125
23	4	0.5	2	0.25
24	2048	2048	64	128
25	4	0.25	0.5	0.25
26	4	0.25	0.5	0.25
27	2048	1024	64	128
28	8	0.5	0.5	128
29	4	0.5	0.5	0.125
30	2048	1024	64	256
31	2048	2048	8	256
32	1024	512	8	256
33	1024	512	8	256
34	8	0.5	0.5	64
36	2048	512	8	512
37	16	0.5	0.5	0.25
38	16	0.5	0.5	0.125

Table 5 continued. Minimal Inhibitory Concentrations (MICs) of ceftriaxon, flocloxacillin, ciprofloxacin and gentamicin against tested MRSA.

Strains no.	Ceftriaxon (µg/ml)	Flocloxacillin (µg/ml)	Ciprofloxacin (µg/ml)	Gentamicin (µg/ml)
39	2048	2048	16	512
40	8	0.5	0.25	0.25
41	2	0.25	0.5	0.25
42	1024	512	32	512
43	8	0.25	16	1
44	8	0.5	2	64
45	8	0.5	16	>2048
46	2048	1024	64	512
47	2048	2048	64	2048
48	1024	512	16	2048
49	1024	512	16	2048
50	>2048	2048	128	0.25

Table 6. Total cell protein patterns of MRSA isolates (n=49).

Patterns no.	Strains no.	Clinical source	No. of strains	% distribution
1	8, 27,28,31, 39, 40	Urine	16	32.65%
	7, 9, 10, 11, 34, 41	Blood		
	3	Vaginal		
	2, 43, 49	Sputum		
2	25, 26, 36, 37, 38, 42	Skin infection	11	22.45%
	44, 45, 46, 47, 48	Blood		
3	12, 13, 14, 16, 17, 23, 24	Urine	8	16.33%
	6	Blood		
4	19, 21, 22, 50	Skin infection	4	8.16%
5	30, 32, 33	Blood	3	6.122%
6	15, 18, 20	Vaginal	3	6.122%
7	1, 4	Wound	2	4.08%
8	5	Sputum	2	4.08%
	29	Urine		
9	15	Vaginal	1	2.04%

hospitalization increase the chance of prolonged household contact and horizontal transmission of MRSA. Thus, admission surveillance culture to those at high-risk would be valuable for early detection of MRSA carriers but still require substantial resources and coordination between health care sectors.

Bacterial infections are associated with worsening of prognosis and increased mortality rates due to precipitation of decompensation among cirrhotic patients [22].

In this work, all HE participants were MRSA-positive and its decolonization (100%) in vancomycin-susceptible [45/49 (91.84%)] patients improved the number of HE survivors

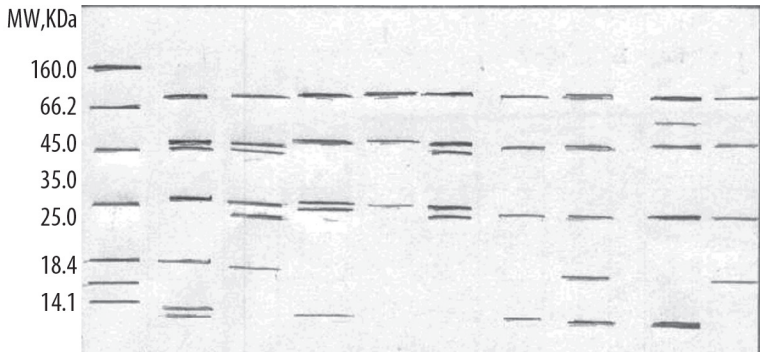

Figure 1. Schematic representation of different total cell protein patterns. Lane M was mid-range molecular weight marker. Lanes 1to 9 are patterns No. 1, 2, 3, 4, 5, 6, 7, 8 and 9 respectively.

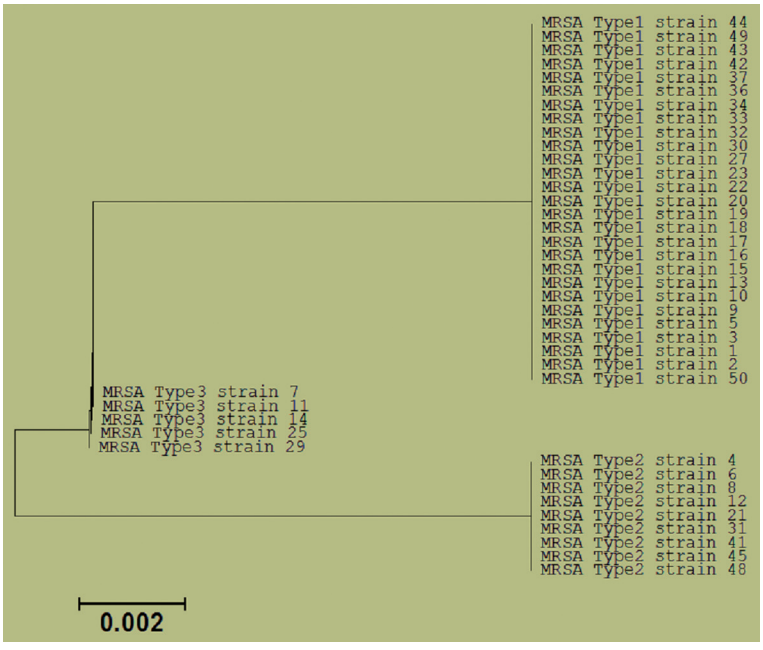

Figure 2. Neighbour – joining tree of *mecA* sequences showing the three different groups of *mecA* amplified from the tested strains.

[35/49 (71.42%)] in the hepatology ICU setting where a high MRSA pool exists.

In the present study, a total of 49 (26.92%) strains were isolated and identified as MRSA strains from 184 *Staphylococcus aureus* isolates from different clinical sources admitted at our ICU. The disc diffusion (oxacillin and cefoxitin) methods were used for MRSA screening. Cefoxitin disc diffusion test give clearer endpoints and are easier to read than tests with oxacillin [23]. According to the interpretation chart of cefoxitin disc diffusion method (30 mcg), all isolates were reported as MRSA with a specificity of (43/49) 87% and distinguished MRSA from MSSA and suggested that oxacillin disc diffusion test can not be considered as an accurate method for detection of MRSA alone [24].

In this study, the sensitivity patterns of MRSA isolates showed that all 49 isolates (100%) from different sources were resistant to cefoxitin, ampicillin, ceftriaxon and cloxacillin (Table 3). In contrast, most isolates were susceptible to vancomycin (91.84%). Results of the resistance pattern were confirmed by determination of the MIC of different tested antimicrobial agents against MRSA isolates as determined by agar dilution method (Table 5). Our data indicate that the detected MRSA isolates were multi-drug resistant with a substantial morbidity and mortality and of course, with a certainly limited choice of therapeutic antibiotic options that would explain how difficult to control and eradicate MRSA-related consequences. Moreover, vancomycin can be reliably effective in most cases and its in-hospital use should be reserved to the highly indicated cases only as suggested by an appropriate culture and sensitivity testing.

Variable sensitivity patterns of all isolates have been demonstrated in different studies that may be attributed to the fact that resistance rates vary from country to country [25]. Thus, an increased clinicians' awareness about the local susceptibility and resistance patterns would be of value in the therapeutic decisions. Moreover, judicious use of antibiotics in serious infections especially, in critically ill patients is strongly advised under guidance of culture and sensitivity testing to overcome the emergence of bacterial antimicrobial resistance and to benefit from the natural "colonization resistance" against MRSA, offered by the commensals' flora that would be lost by the empirical use of broad spectrum antibiotics.

Most strains of MRSA as well as MSSA are now resistant to quinolones, including its new generations [26]. Resistance

Table 7. Distribution of different types of *mecA* among the tested strains.

Strain #	Type 1	Type 2	Type 3
1			
2			
3			
4			
5			
6			
7			
8			
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10			
11			
12			
13			
14			
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50			

to quinolones results from stepwise acquisition of chromosomal mutations. Additionally, increased expression of the NorA multi-drug resistance efflux pump in *Staphylococcus aureus* can result in low-level quinolone resistance [27]. The essential mechanism of oxacillin/methicillin resistance in staphylococci is production of an additional low affinity penicillin-binding protein PBP2a, encoded by the chromosomal *mecA* gene [28]. Detection of *mecA* gene or its product, penicillin binding proteins, is considered the gold standard for MRSA confirmation [29,30]. Rajan et al. [31] mentioned the usefulness of PCR for rapid MRSA detection in a busy ICU where MRSA is endemic.

In the present study, PCR amplification of *mecA* gene indicate that only 41 samples amplified with PCR where samples No. 24, 26, 28, 38, 39, 40, 46 and 47 gave negative results. Comparing PCR rapidity with the traditional methods, 24–48 hrs incubation on MSA is needed, then testing for catalase, coagulase require,at least, 4 hours if plasma is used, then detection for resistance with oxacillin disc requires an overnight incubation in broth and another incubation for 24 hrs for culturing on nutrient agar. These steps are time-consuming and relatively inaccurate and its use, to some degree, is limited to the local availability, economic resources and personal qualifications. In fact, MRSA detection needs rapidity and accuracy especially, for those at high risk of hospital infections. Thus, despite its expenses, PCR still the technique of choice for MRSA identification and proved to be an accurate, rapid, time-saving technique to recognize MRSA than the phenotypic method.

Conclusions

Our data revealed an increased prevalence of multi-drug resistant MRSA among the tested isolates where PCR was of the best choice for their rapid and accurate detection. Admission surveillance culture to those at high-risk for early detection of MRSA carriers is highly suggested and a comprehensive infection control program should be implemented for appropriate MRSA management.

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Experimental & Clinical Hepatology

Received: 2012.02.01
Accepted: 2012.02.27
Published: 2012.06.01

Chlamydial antigen and nucleic acid detection in liver biopsies from patients with chronic cholelithiasis

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B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
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Source of support: Lycotec Ltd., United Kingdom

Summary

Background: It is known that chlamydial species can propagate in hepatocyte cell lines. Moreover, some clinical cases of chlamydial infection involve liver abnormalities. This study was to clarify whether chlamydial markers (protein and nucleic acids) could be detected in liver biopsies from patients with calculous cholecystitis.

Material/Methods: Liver biopsies were obtained from 39 patients during cholecystectomy and analyzed with immunohistochemical, nucleic acid amplification and serological protocols. Liver specimens from 8 trauma victims served as controls.

Results: It was shown that from 39 patients with cholecystitis 19 gave considerable signal generated by antibodies against *C. trachomatis* (15 patients) or *C. pneumoniae* (4 patients). 10.2% (4/39) of the samples contained detectable 16S rRNA genomic sequence from *C. pneumoniae* while amplifiable fragments of 16S rRNA and pLGV cryptic plasmid from *C. trachomatis* were found in 20.5% (8/39) of DNA specimens. The control group had a zero detection rate for chlamydial genetic markers in the liver. Simultaneous detection of genetic and immunohistochemical markers validated by positive serological status took place in a very limited number of the patients (4 cases for *C. trachomatis* and 2 cases for *C. pneumoniae*). Moreover, it was shown that *C. trachomatis* and *C. pneumoniae* can efficiently propagate in freshly isolated rat primary hepatocytes forming infectious progeny.

Conclusions: Identification of chlamydial markers in liver biopsies along with the ability of the chlamydial pathogens to propagate in native hepatocytes may suggest the possible involvement of chlamydial species in inflammatory hepatobiliary disease.

Key words: ***C. trachomaris*** • ***C. pneumoniae*** • **liver biopsies** • **cholelithiasis** • **PCR** • **immunohistochemistry**

Full-text PDF: http://www.expclinhep.com/fulltxt.php?ICID=997405

Word count: 4043
Tables: 1
Figures: 4
References: 47

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Background

C. trachomatis and *C. pneumoniae* are two major human pathogens from the genus Chlamydia [1]. It was postulated long ago that chlamydial species have a strict tissue tropism. *C. pneumoniae* preferentially targets epithelial cells of the respiratory system, whereas *C. trachomatis* is known to infect epithelial cells of the urogenital tract and conjunctivae [2,3]. However, some chlamydial strains can easily penetrate submucosal membrane and infect lymphocytes, spreading through human body via regional lymphatic and blood vessels [4]. Among the different serological variants of *C. trachomatis* at least the L1, L2 and L3 serotypes are believed to have distinct invasive properties [5]. In contrast, other serovars (ocular A-C and genital D-K) restrict their propagation to mucosal epitheliocytes [5,6]. The invasive properties of *C. trachomatis* can explain the appearance of the pathogen in some extragenital tissues and fluids of the human body – liver, synovial exudates, ascitic fluid and respiratory secretion fluid [7–10].

Invasiveness is also an unquestionable feature of *C. pneumoniae*. Its isolates have been obtained from respiratory secretion fluid as well as nasal, tracheal and lung tissue of patients [11–13]. There are numerous reports on detection of *C. pneumoniae* in atherosclerotic plaques [14], myocardium [15], brain [16] cerebrospinal fluid [17] and joints [18].

Chlamydial species target different organs since there are a remarkable variety of eukaryotic cells supporting chlamydial growth. Chlamydia can efficiently propagate in mononuclear cells [18] as well as in astrocytes, microglia, muscle cells and myocardiocytes [19–23]. Thus, the striking ability of chlamydial pathogens to accomplish their developmental cycle in non-epithelial cells is likely to be a crucial determinant for generalization of chlamydial infection *in vivo*.

Among cells recently discovered to be capable of supporting the chlamydial life cycle are hepatocytes [24,25]. We have recently reported that *C. trachomatis* and *C. pneumoniae* can efficiently propagate in a human hepatoma cell line – HepG2 cells. Chlamydial growth in a HepG2 cell line affects transcription of some liver-specific genes and leads to the formation of infectious progeny [26]. However, immortalized hepatoma cell lines have a very remote resemblance to the phenotype of "native" hepatocytes due to irreversible dedifferentiation [27]. Therefore, neither the effect of infectious agents on hepatic function nor their developmental cycle in liver can be accurately studied using hepatoma cells [28]. Primary hepatocytes whose phenotype can be efficiently preserved in the short term [29] might be a much better option for acute *in vitro* experiments with hepatotropic pathogens.

Our recent paper also originates from the assumption that molecular markers of chlamydial pathogens might be detected in human liver biopsy material obtained from patients with inflammatory hepatobiliary disease.

Here we report that *C. trachomatis* and *C. pneumoniae* can efficiently propagate in freshly isolated rat primary hepatocytes forming infectious progeny. Both pathogens can be detected in liver biopsies obtained from patients with cholelithiasis using specific immunochemistry and nucleic acid amplification protocols.

Material and Methods

Patients

The clinical work was conducted at the Razumovsky Medical University and Institute of Cardiology (Saratov, Russian Federation) from January 2007 to January 2008. The study protocol was approved by the local Ethical Committee. All patients were informed about the purpose of the study and have given written consent regarding participation in the study. The major group of the study included 39 patients who underwent open cholecystectomy due to symptomatic chronic calculous cholecystitis (mean age 52.4±6.2; range 38–64 years; 21 females, 18 males). Liver biopsy specimens were obtained during surgery from the hepatic areas adjacent to the gall bladder. Serum specimens were collected from all patients before surgery and stored at –80°C for retrospective determination of antibodies specific to *C. trachomatis* and *C. pneumoniae* in addition to PCR analysis. The study did not interfere with pre-operative therapeutic options or have an impact on post-operative treatment chosen by physicians for each consenting individual. All patients included in the study were negative for features of pelvic inflammatory disease and Fitz-Hugh-Curtis syndrome.

Control specimens (liver and blood) were collected using aseptic technique from 8 trauma victims with uncompromised medical anamnesis within 12 hours of death (aged from 29 to 53 years, 4 females, 4 males). No macro-microscopic evidence of cholelithiasis or other hepatobiliary pathology has been found among specimens added to the control group.

Specimen handling

All specimens were collected in the operating room under sterile conditions. Liver specimens approximately 4×4 mm in length were placed in microcentrifuge tubes. Transport vials were sealed in the operating room and opened only in the laminar air flow safety cabinet at the Department of Medical Microbiology in the Gamaleya Institute of Epidemiology and Microbiology (Moscow, RF). All specimens were kept at –70°C until processing. Dissected tissue was homogenized using a sterile glass grinder. Chromosomal DNA was extracted by the cetyltrimethylammonium bromide (CTAB) method according to the DNA Miniprep protocol of Wilson (30). This method is known to remove complex polysaccharides interfering with PCR amplification.

Reagents and bacteria

All reagents were purchased from Sigma-Aldrich unless mentioned otherwise. The *C.trachomatis* strain L2/Bu434 and the *C. pneumoniae* strain *Kajjani-6* were used as reference cultures. Both of them were kindly provided by Prof. P. Saikku (University of Oulu, Finland). *C. pneumoniae Kajaani-6* strain was propagated in *Mycoplasma*-free HL cells whereas *C. trachomatis Bu434* strain was propagated in *Mycoplasma*-free McCoy cells grown in RPMI-1640 medium supplemented with 2 mM l-glutamine (Invitrogen), 5% fetal bovine serum, 50 μg/ml of gentamicin sulfate and 1 mg/ml of cycloheximide. Infectious elementary bodies were isolated [31] from McCoy cells by sonication, washed in phosphate buffered saline, purified by Renografin gradient centrifugation and

kept frozen at –80°C in SPG buffer (pH 7.2; 250 mM sucrose, 10 mM sodium phosphate, 5 mM l-glutamic acid). Chlamydial titers were determined by infecting host cells with 10-fold dilutions of thawed stock suspension.

Bacteriological assay

After overnight transportation biopsy specimens were cut into ~100 mg segments and homogenized separately with a Heidolph Silent Crusher M (Germany) in 1 ml of RPMI-1640 at 4°C. Equal parts of the resulting suspensions were used for PCR and cell culture. Infection of the host cell monolayers (HL and McCoy cells) was performed by centrifugation of 24 well plates at 1500 g for 30 min. Supernatants were replaced with fresh RPMI-1640 containing 1 μg/ml cycloheximide and plates were incubated at 37°C with 5% CO_2 for 3 days. Cells were harvested for DNA extraction. Chlamydial growth was evaluated by comparison of bacterial loads in inocula and harvested monolayers by TaqMan-PCR.

Serological evaluation

Chlamydial antibody titers (IgG, IgM and IgA) were measured according to the standard microimmunofluorescence (MIF) protocol [32]. In brief, chlamydial particles grown and purified from HL (*C. pneumoniae*) or McCoy cells (*C. trachomatis*) were filtered and resuspended in 0.02% formalin in Dulbecco solution. Bacterial suspensions normalized in protein content were kept frozen at –80°C until the assay was performed. Chlamydial antigens were spotted in a 15-circle area on glass slides (ICN Biomedicals, UK), dried and fixed with acetone. Diluted sera and anti-human isotype-specific FITC-labeled antibodies were applied to the glass slides. For *C. trachomatis* IgG titers ⩾1:64 or a collective increase in IgG ⩾1:64 and IgM (or IgA) ⩾1:8 were considered as evidence of positive serological status. *C. pneumoniae* IgG titers ⩾1:128 alone or IgG ⩾1:64 combined with IgM (or IgA) ⩾1:8 were assumed to witness the seropositivity of the patients.

In addition, IgG titers specific to *C. trachomatis* cHSP60 protein were measured in serum specimens using a ChlamiBest cHSP60-IgG kit (Vector-Best Inc, RF).

Immunohistochemistry

Deparafinized and rehydrated 7–10 μm liver sections were blocked in PBS with 1% FCS overnight at 4°C. Monoclonal antibody against lipopolysaccharide of *C. trachomatis* or polyclonal antibody specific to the major outer protein (MOMP) of *C. pneumoniae* (both from NearMedic, RF) were used for immunohistochemistry analysis. After 2 hours incubation with FITC-labeled primary antibodies (5 μg/ml, 37°C) the sections were washed in PBS 3 times and analyzed using a Nikon Eclipse 50i microscope.

Assessment of infective progeny

In order to assess the infective progeny accumulation in rat primary hepatocytes after a 48 hour cultivation period, infected hepatocytes were harvested, frozen and thawed, as described elsewhere. Serial dilutions of lysates were inoculated onto monolayers of HL cells or McCoy cells to verify the growth of *C. pneumoniae* or *C. trachomatis* respectively. The plates were centrifuged for 0.5 hour at 1500 g. The infected cells were visualized with anti-chlamydial genus-specific monoclonal FITC-labeled antibodies (NearMedic, RF) after 48 hours.

Primary hepatocyte isolation

Primary hepatocytes were isolated from Spraque-Dawley rat liver of non-fasted rats by the collagenase perfusion method as described [33]. Animals 6–8 wks old were obtained from Pushino Animal Breeding Facility (Moscow, RF) and kept in the animal facility in compliance with the "Declaration of Helsinki and Guiding Principles in the Care and Use of Animals" under an approved protocol at the Gamaleya Institute for Epidemiology and Microbiology (Moscow, RF). Livers of halothane-anesthetized rats were perfused *in situ* through the portal vein with warmed (37°C) Liver Perfusion Medium and later with Liver Digest Medium (Gibco/BRL, UK). Livers were excised and the hepatic capsule disrupted with needles in Digest Medium. The resulting cell suspension was filtered and washed twice by low-speed centrifugation (20 g, 3 min, 4°C) in ice-cold DMEM with 10% FCS and penicillin/streptomycin (100 μg/ml each). Remaining non-hepatic cells were eliminated by pre-absorption on 100 mm plastic dishes at 37°C for 20 min. Viability and purity of unattached cells were determined before plating. Cell suspensions with a viability rate ⩾90% in the trypan blue exclusion test were used. Purified primary hepatocytes were plated onto BD BioCoat™ collagen-coated cover slips. After 3 hours attachment cell monolayers were washed with serum-free DMEM containing 0.4% glucose and 2 μg/ml cycloheximide. 6-well plates with inserted cover slips were infected with *C. trachomatis* or *C. pneumoniae* by centrifugation at 1500 g for 30 min at multiplicity rate 2. After incubation at 37°C for 48 hours (95% O_2, 5% CO_2) cover slips were fixed with acetone. Permeabilized cells were stained by direct immunofluorescence using anti-chlamydial genus-specific FITC – conjugated monoclonal antibody (NearMedic, RF). Inclusion-containing cells were visualized using a Nikon Eclipse 50i fluorescence microscope at ×1350 magnification.

DNA isolation

Extraction of total nucleic acids was conducted with a NucliSENS® easyMAG® automated system (BioMerieux Inc., Netherlands). Briefly, ~50 mg of biopsy specimens were homogenized with 1 ml of lysis buffer (BioMerieux) containing 0.25 mg/ml proteinase K (Promega, USA). After 3 hours of incubation at 55°C digested specimens were loaded onto the NucliSENS® easyMAG® platform. Loading of samples, reagents and disposables were the only manual steps during the DNA extraction procedure using the NucliSENS® easyMAG® platform. Up to 24 samples were analyzed in one BioMerieux automated run. DNA was eluted from the cartridges with 50 μL of BioMerieux elution buffer. Bacterial load in serum specimens and bioptates is shown below in genome equivalents of per ml of serum or in genome equivalents of the pathogens referred to 10^6 copies of eukaryotic β-actin (liver specimens). Calibration standards were prepared using amplified fragments of 16S rRNA from *C. pneumoniae*, 16S rRNA and pLGV440 from *C. trachomatis*, or eukaryotic β-actin and cloning them in the pGEM-T plasmid vector (pVU56) using a TA cloning kit (Invitrogen, San Diego, CA) similarly to Broccolo's protocol [34].

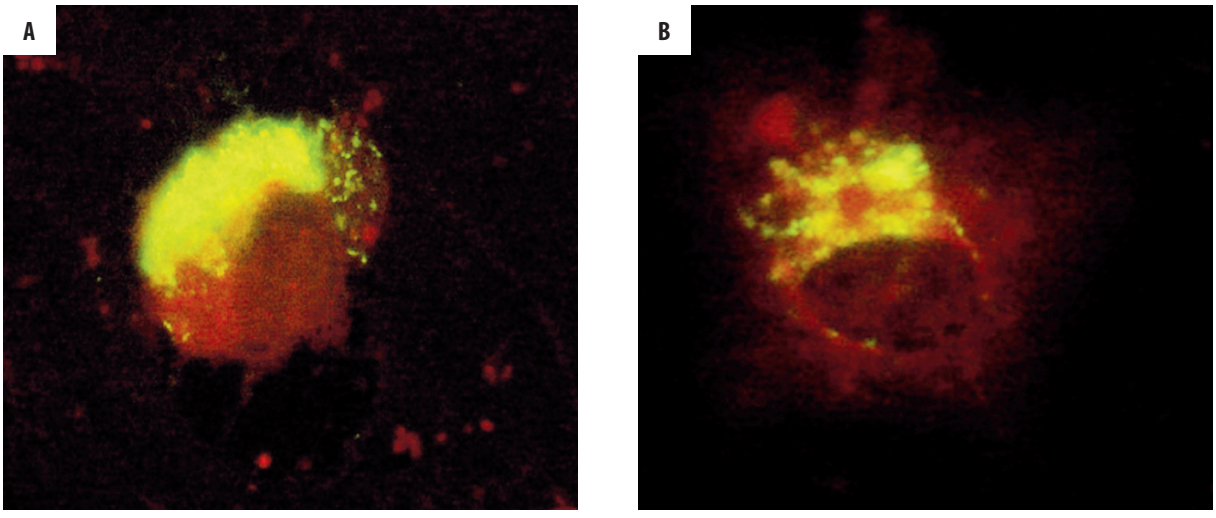

Figure 1. Imminofluorescent staining in rat primary hepatocytes infected with *C. trachomatis* (**A**) and *C. pneumoniae* (**B**). Rat primary hepatocytes we isolated, plated and infected with *C. trachomatis* (**A**) and *C. pneumoniae* (**B**) and stained with FITC-labeled genus-specific antibody against chlamydial lipopolysaccharide as described in the "Material and Methods". The slides were visualized and photographed using 90 × immersion objective.

Quantitative TaqMan-PCR

For quantification purposes, real-time PCR for 16S rRNA of *C. pneumoniae* and for 16S rRNA and cryptic plasmid of *C. trachomatis* was conducted. PCR primers and TaqMan probes for 16S rRNA *C. pneumoniae* (GenBank accession number), for 16S rRNA of *C. trachomatis* (GenBank accession number AM884176) and cryptic plasmid of *C. trachomatis* (GenBank accession number X06707.3) were designed using Primer Express Software (Applied Biosystems, Foster City, CA, USA) and synthesized by Syntol Inc. (Moscow, RF). Designed primers and TaqMan probes were: for 16S rRNA of *C. pneumoniae* forward primer, 5'-GGTCTCAACCCCATCCGTGTCGG-3'; reverse primer, 5'-TGCGGAAAGCTGTATTTCTACAGTT-3'; and TaqMan probe, ROX-TCCAGGTAAGGTCCTTCGCGTTGCATCG-BHQ2; for 16S rRNA of *C. trachomatis* forward primer, 5'-GGCGTATTTGGGCATCCGAGTAACG-3'; reverse primer, 5'-TCAAATCCAGCGGGTATTAACCGCCT-3'; and TaqMan probe R6G-TGGCGGCCA ATCTCTCAATCCGCCTAGA-BHQ2; for cryptic plasmid of *C.trachomatis* forward primer, 5'-GGGATTCCTGTAACAACAAGTCAGG-3'; reverse primer, 5'-CCT CTTCCCCAGAACAATAAGAACAC-3'; and TaqMan probe ROX-CTCCCAGAG TACTTCGTGCAAGCGCTTTGA –BHQ2. The predicted sizes of the generated PCR products were 194 bp, 316 bp and 206 bp respectively. An additional BLAST search analysis was conducted to ensure specificity of the primers and probe. Real-time PCR was performed with the iCycler IQ system (Biorad, USA). 2 µl of the extracted DNA was analyzed with the PCR mixture in a total volume of 25 µl. The PCR mixture consisted of 10 mM Tris (pH 8.3), 50 mM KCl, 1,5 mM $MgCl_2$, 200 µM of each dNTPs, 2,5 U of Thermostar *Taq* DNA polymerase (Syntol, Moscow, RF); and 5 pmol of both forward and reverse primers and 3,5 pmol probe. The real-time PCR run was 10 min at 95°C, and 50 repeats of 20 sec at 95°C and 50 sec at 62°C. All samples were analyzed in triplicates. A sample was considered positive if three out of three assay results were positive in the triplicate test and if the average value for the PCR runs was greater than or equal to 1.0.

The cycle threshold (C_T) values, defined as the number of cycles at which the fluorescence of the reporter dye first exceeds the calculated background level, were automatically estimated by the instrument for each reaction. C_T values of specimens were plotted against calibration standards of cloned DNA fragment. Gel mobility of amplification products and their sequencing were performed to confirm identity of pathogens in some positive specimens. Specimens with cycle threshold (*CT*) values exceeding 35 were considered as negatives.

Results

Chlamydial infection in rat primary hepatocytes

Figure 1 shows immunofluorescence (IF) in rat primary hepatocytes after inoculation with *C. trachomatis* (Figure 1A) and *C.pneumoniae* (Figure 1B). IF signal became visible after 20 hours of the postinfection period when some parts of the hepatocyte perinuclear area started to appear slightly opalescent with punctuate and granular structures. Typical inclusion morphology started to emerge in the 48 hour hepatocyte cultures. *C. trachomatis* inclusions were large and had homogeneous IF staining resembling those traditionally observed in McCoy cells. In contrast, multiple granular particles were seen within the *C. pneumoniae* inclusions. These were smaller and had less intense IF signal as compared to *C. trachomatis* infected cells. Formation of chlamydial inclusion bodies within hepatocytes led to nucleus dislocation especially in the case of *C. trachomatis* infection. At later stages (72 h) infected hepatocytes were enlarged, poorly attached and tended to come off the collagen-covered slips. Some of the cells appeared to be ruptured with most of the chlamydial endosomes released. Under the conditions used, successful chlamydial infection has been observed in ~50% of primary hepatocytes regardless of the pathogen type. Lysates obtained from primary hepatocytes infected with chlamydial pathogens were capable of inducing new rounds of chlamydial infection and specific immunostaining in McCoy (*C. trachomatis*) and HL (*C. pneumoniae*) cells (results not shown).

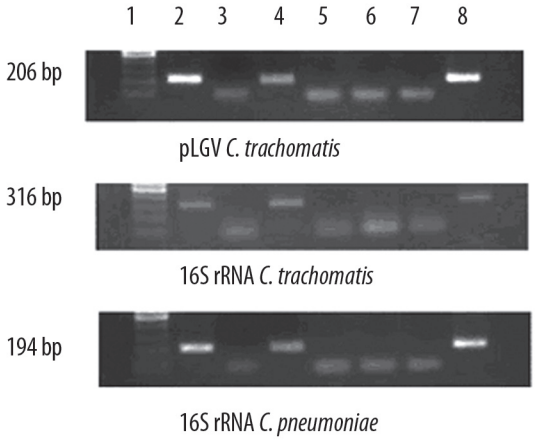

Figure 2. Amplicon size verification by gel electrophoresis. Recovery of PCR products in amplification reactions with different primers (pLGV C. trachomatis, 16 S rRNA C. trachomatis and 16 S rRNA C. pneumoniae) using DNA extracted from liver biopsies and rat primary hepatocytes infected with the chlamydial pathogens: 1 – molecular size standards, 2 – Liver from positive patient V, 3 – Liver from negative patient K, 4 – Primary hepatocytes infected with C. trachomatis or C. pneumoniae, 5 – uninfected primary hepatocytes, 6 – PCR negative control, 7 – DNA isolation control and 8 – positive control (plasmids with corresponding specific inserts). All procedures were performed as described in the "Material and Methods".

Moreover, as can be seen in Figure 2 we were able to amplify specific chlamydial genetic markers – pLGV (*C. trachomatis*) and 16S rRNA (*C. trachomatis* and *C. pneumoniae*) in DNA extracted from primary rat hepatocytes infected with each particular chlamydial pathogen.

Serological evaluation of the patients

Table 1 shows the results of serological status of the patients. As can be seen, IgG seropositivity for *C. pneumoniae* seems to be quite a common finding affecting 69.2% of the patients with cholelithiasis. In contrast, only 20.5% of the patients had detectable IgG levels for *C. trachomatis*. Seroprevalence of anti-HSP60 IgG specific to *C. trachomatis* was in good agreement with the IgG detection rate. Control serum specimens showed a remarkably lower incidence of seropositivity to chlamydial antigens. Isotype-specific response has been mostly limited to the IgG class of immunoglobulins in both groups.

Immunohistochemistry

Immunohistochemistry analysis of liver bioptates revealed that from 39 patients with chronic cholecystitis 19 have had considerable signal originated by preincubation of the sections with antibodies against *C. trachomatis* (15 patients) or antibodies against *C. pneumoniae* (4 patients). Inclusions visualized with antibody against *C. trachomatis* were numerous and large (Figure 2), while inclusions seen in the sections preincubated with *C. pneumoniae* – specific antibodies were much smaller and less abundant (Figure 3). Among control liver biopsies positive immunostaining for *C. pneumoniae* was not detected in any specimens and signal generated with *C. trachomatis* antibodies was not seen in any specimens either. In all sections immunohistochemistry signal had no clear association with hepatic vascular topography.

PCR analysis

The nucleic acid amplification protocol used in our study revealed that liver DNA obtained from 10.2% (4/39) of patients with cholelithiasis contained detectable 16S rRNA genomic sequence of *C. pneumoniae*. No positives were detected in the control group.

On the other hand, amplifiable fragments of 16S rRNA and pLGV cryptic plasmid of *C. trachomatis* were found in 20.5% (8/39) of DNA specimens extracted from liver biopsies of cholelithiasis patients. Simultaneous detection of genetic and immunohistochemical markers was found in 12.8% (5/39) of patients. The control group had a zero detection rate of chlamydial pathogen genetic markers in the liver. Amplification products were routinely analyzed in gel electrophoresis with all relevant controls (Figure 4). Amplicons derived from RT-PCR reactions with hepatic DNA matched up in their gel mobility to the amplification products derived from reference cultures and primary rat hepatocytes infected with chlamydial pathogens. Randomly chosen positive PCR reactions (5 total) were subjected to sequencing and confirmed the identity of amplicons and the specificity of PCR analysis.

All attempts to quantify bacterial load in liver tissue were complicated to some extent by variations in triplicates and some differences in β-actin counts in hepatic DNA specimens. However, our best estimate of the bacterial load for chlamydial pathogens in the liver tissue is very low with a median value for *C. trachomatis* ~8.5×10^2 copies/1×10^6 copies of β-actin. The corresponding value for *C. pneumoniae* was ~5.5×10^3 copies/1×10^6 copies of β-actin.

Bacteriological assay

We failed to obtain culturally retrievable isolates of the chlamydial pathogens from the liver specimens. PCR quantification of the genetic markers in the inocula and the post-cultivation DNA specimens showed no significant difference in the amounts of pLGV cryptic plasmid for *C. trachomatis*. In 4 cases (cholelithiasis group) there was a measurable increase in the amount of 16S rRNA for *C. pneumoniae* after cultivation of liver biopsy material in HL cells.

Discussion

Liver cell heterogeneity predetermines the remarkable diversity of hepatic functions. Parenchymal cells (hepatocytes) as well as non-parenchymal cells (Kupffer cells, stellate cells and hepatic endothelial cells) are reported to be involved in the innate immune response to different pathogens. Yet their involvement in the pathogenesis of chlamydial infection remains unknown. As we have published previously [25,26], *C. trachomatis* and *C.pneumoniae* can efficiently propagate in an immortalized hepatic cell line (HepG2 cells). However, HepG2 cells do not display the whole array of hepatic markers and functions. Hepatoma cell lines are known to have abnormal gene expression, uncontrolled proliferation, anomalous signaling and atypical receptor turnover [35]. Therefore, it was essential for us to explore

Table 1. Serological variables.

Group	MIF – *C. pneumoniae*			MIF – *C. trachomatis*			ELISA *C. trachomatis* HSP60 IgG
	IgG	IgM	IgA	IgG	IgM	IgA	
Control (n=8)	2	0	0	1	0	0	0
Cholelithiasis (n=39)	27	0	1	8	0	0	9

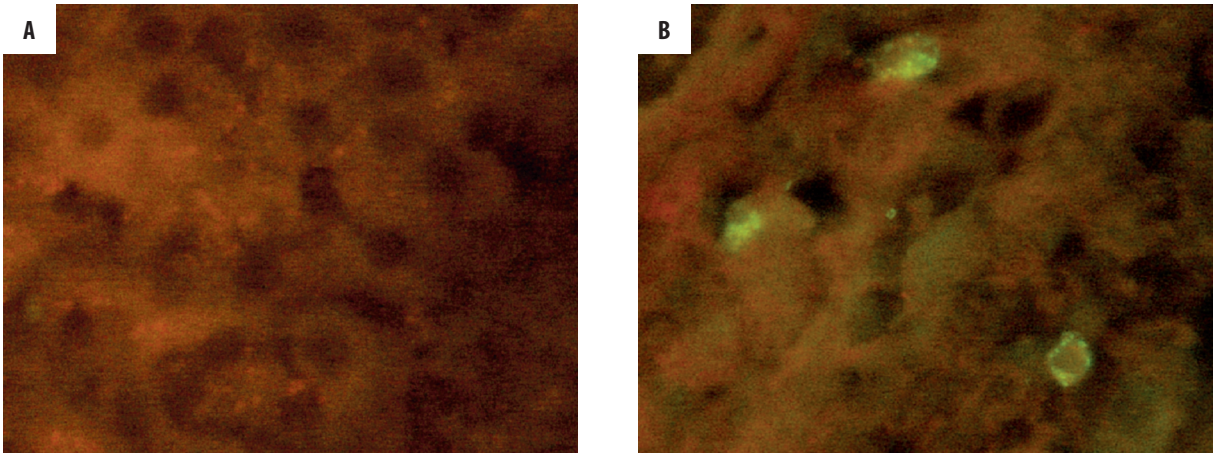

Figure 3. Immunohistochemical staining of liver biopsy with non-immune IgG (**A**) *C. trachomatis* – specific antibody (**B**) liver biopsies were obtained, processed and hepatic sections were immuno-stained with non-immune IgG (**A**) and monoclonal antibody against monoclonal antibody against lipopolysaccharide of *C. trachomatis* (**B**) as described in the "Material and Methods".

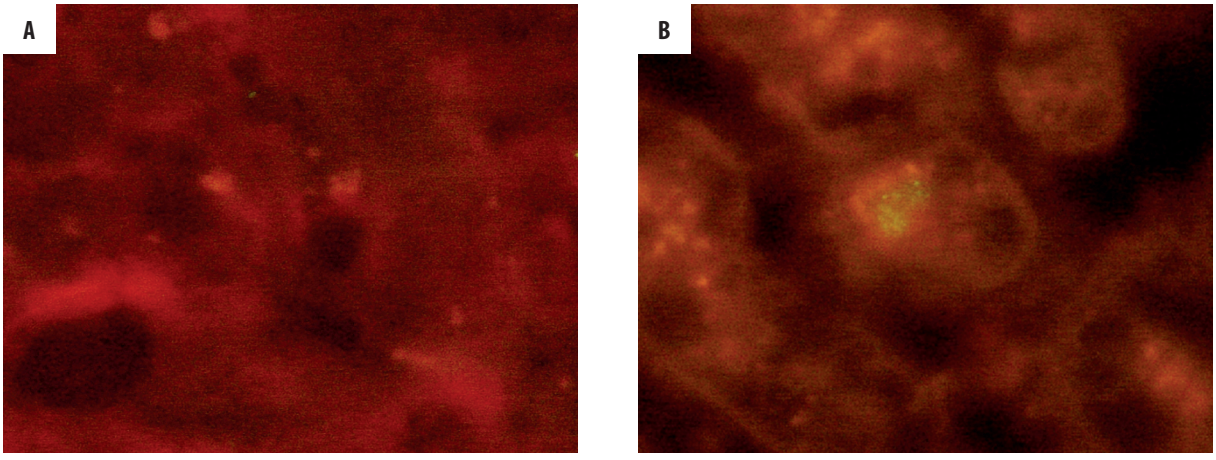

Figure 4. Immunohistochemical staining of liver biopsy with *C. pneumoniae* antibody. Liver biopsies were obtained, processed and hepatic sections were immuno-stained with non-immune IgG (**A**) and polyclonal antibody specific to the major outer protein (MOMP) of *C. pneumoniae* (**B**) as described in the "Material and Methods".

at this stage whether chlamydial biovars could propagate in freshly isolated primary hepatocytes whose phenotype remains relatively well preserved on a collagen matrix in the short term [36].

Herein we show that freshly isolated rat hepatocytes provide perfect support for the full developmental cycle of both chlamydial pathogens. Primary hepatocyte infection caused by *C. trachomatis* or *C. pneumoniae* is productive and leads to the formation of infective progeny. Overall dynamic and morphological features of the chlamydial infectious cycle in the primary hepatocytes remarkably resemble those seen in the classical cell lines used for chlamydial research (McCoy and HL cells).

Secondly, and most importantly, in the recent work we have shown that chlamydial antigens as well as chlamydial genetic markers can be detected in the human liver of patients suffering from cholelithiasis. Immunostaining analysis for chlamydial antigens is known to produce a high positivity rate among patients (37) which was the case for the liver sections in our study. A smaller number of the patients were

positive in TaqMan PCR. It is essential that the PCR signal in human liver was attributable to the chlamydial primary rRNA sequences which are indicative of active infection since extrinsic chlamydial DNA is rapidly degraded by restriction endonucleases [37,38]. There was no reasonable agreement between the detection rate of chlamydial markers in liver biopsies and serological status of the patients especially in case of *C. pneumoniae.* Simultaneous detection of genetic and immunohistochemical markers validated by positive serological status took place in a rather very limited number of the patients (4 cases for *C. trachomatis* and 2 cases for *C. pneumoniae*).

Even though our results may present an obvious step forward in the understanding of chlamydial diseases, there are some significant limitations in the relevance of our data to clinical practice. To begin with, although immunostained cells in the hepatic sections noticeably resemble hepatocytes, our current results do not disclose clearly what types of hepatic cells have positive immunostaining for chlamydial antigens in liver tissue. Theoretically, besides hepatocytes, whose ability to support chlamydial growth is shown in our previous and recent work, some other hepatic cells such as Kupffer and endothelial cells can bear viable chlamydial pathogens [39,40]. Although immunohistochemical study on cell type identification is now under way in our lab and might be extremely valuable for future therapeutic strategies, this question is of secondary significance to the current paper. It is rather more important to us to report at this stage the fact that hepatic biopsies may contain chlamydial immunohistochemical and genetic markers. This suggests that the liver might be a target organ for chlamydial infection, harboring active pathogens in the human body.

Another concern arises from the very low values for bacterial load in biopsy material revealed by both the nucleic acid amplification protocol and the immunohistochemistry method. However, a low copy number is rather a common problem for human specimens. Detection of *C. pneumoniae* in atherosclerotic plaque often approaches the sensitivity limit of the RT-PCR assay [41]. A low number for *C. trachomatis* bacterial load has been also reported for synovial fluid from patients with reactive arthritis [42].

At first sight there is a worrisome discrepancy between infection rate seen in primary rat hepatocytes and liver biopsy specimens from cholelithiasis patients. However, such disagreement may originate from, the use of a centrifugation protocol to infect primary hepatocyte monolayers. Although centrifugation remains a main conventional tool in infecting cultured cells with chlamydial species, such a highly artificial procedure has no analogy *in vivo*. Centrifugation may force attachment of chlamydial particles to the cell membrane despite low affinity of the pathogen for the host cell.

However, our previous data revealed that there might be a highly-specialized and exclusive mechanism for chlamydial entry to the hepatocytes. We have shown that *C. trachomatis* and *C. pneumoniae* bind to ApoB-containing lipoproteins boosting infectivity rate of chlamydial particles in a hepatoma cell line. As a result, LDL and VLDL receptors can facilitate the entry of *Chlamydia* into hepatocytes [25]. Therefore, even random attachment of chlamydial particles to the cell membrane is likely to be followed by receptor-facilitated entry of the pathogens into the hepatocytes. We have assumed previously [25] that abnormalities of cholesterol homeostasis associated with the increase of ApoB-containing lipoproteins (VLDL and LDL) may promote enhanced uptake of chlamydial particles in the liver. In this regard it becomes essential that intrahepatic cholestasis and cholelithiasis are known to be accompanied by dyslipidemia with an increased level of ApoB [43]. Thus, it is conceivable that the appearance of chlamydial markers in hepatic biopsies of patients with cholelithiasis takes place due to pro-atherogenic changes in plasma lipoprotein profile.

Moreover, our results might reflect a possible link between chlamydial infection and liver diseases. Although additional studies are required to back up such an assumption, there are a small number of clinical observations supporting our finding. In particular, immunohistochemical detection of *C. pneumoniae* and/or *C. trachomatis* has been reported previously in liver specimens from patients with prolonged fever, Fitz-Hugh syndrome and biliary cirrhosis [44–46]. In broader terms, a possible role of *C. pneumoniae* in the pathogenesis of the primary biliary cirrhosis has been extensively discussed previously [46]. We also realize that our results do not establish any causal relationship between hepatobiliary diseases and chlamydial pathogens. Fulfillment of Koch's postulates is required to make such a claim [47]. Nevertheless, any assumption regarding the possible role of chlamydial species in the pathogenesis of cholelithiasis would be premature with the exception of their likely contribution to the inflammatory background of the disease.

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Experimental & Clinical Hepatology

Received: 2012.03.09
Accepted: 2012.03.15
Published: 2012.06.01

Human RNA methylating enzyme DNMT2 gene expression is downregulated in chronic hepatitis B virus infection

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Summary

Background: Epigenetic regulation of viral replication through cytosine methyl transferases (DNMTs) appeared as a major mode of anti viral defense mechanism.The aim of the study was to assess host DNMT gene expressions in various stages of hepatitis B virus(HBV) infection in relation to viral DNA and surface antigen(HBsAg) concentration in sera.

Material/Methods: DNMT 1,2,3a and 3b mRNA expression in peripheral blood mononuclear cells (PBMCs)of109 HBV infected patients grouped in to acute (AHB), inactive carriers (IC), chronic (CHB), liver cirrhosis (LC) and hepatocellular carcinoma (HCC) was evaluated by reverse transcription polymerase chain reaction (RT-PCR) assay. Serum HBV DNA and HBsAg concentrations (qHBsAg) were measured by real time PCR and sandwich enzyme-linked immunosorbent assay respectively. Forty one voluntary blood donors served as controls.

Results: DNMT 1gene expression was significantly reduced in CHB(p=0.04) while DNMT 3a (p=0.004) and 3b(p=0.0007) expressions were significantly lower in LC only.DNMT2 gene expression was significantly declined ($p<0.01$) in all the desease states compared to controls.HBsAg level had no effect on expression of DNMT genes. Higher level of HBV DNA was associated with reduced expression of DNMT2 having a significant inverse correlation ($r=-0.577, p<0.001$).

Conclusions: The results showed significant downregulation of RNA methylating enzyme DNMT2 mRNA in CHB infection and LC. Considering DNMT2 as a major component of primitive antiviral defense mechanism, the influence of higher viral load in suppressing DNMT2 gene expression might help persistence of HBV in chronic infection.

Key words: DNMT • gene expression • PBMC • HBV infection • viral load

Full-text PDF: http://www.expclinhep.com/fulltxt.php?ICID=997406

Word count: 2851
Tables: 1
Figures: 5
References: 32

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Background

Hepatitis B virus (HBV) infection is a major cause of morbidity and mortality with more than 350 million chronic carriers worldwide [1]. Being considered as one of the most contagious diseases, HBV, apart from its persistence in inactive carriers (IC), is known to increase the risk of chronic hepatitis (CHB), liver cirrhosis (LC) and the development of hepatocellular carcinoma (HCC) in infected subjects [1,2]. While the acute form of the disease is supposed to be self resolved, chronicity of HBV infection among a group of patients often presented with variable outcomes. It has been suggested that the various outcomes of CHB were likely due to the poor immune status or modulation of cell mediated immune response of the host by the viral componenets [3]. The interplay of the host immune response and the viral replication ability is a prime determinant of the likelihood of liver injury, its intensity, and progression to cirrhosis [4,5].

Apart from HBV specific immune suppression and existence of stable forms of HBV i.e. covalently closed circular DNA (cccDNA), infection of immunologically privileged sites like peripheral blood mononuclear cells (PBMCs) has been attributed as potential cause of HBV persistence as well as reinfection [6].

HBV acts as a stealth virus by not inducing innate immune response of the host [7] but counteract the adaptive immune response which is known to be activated by the virus [8]. In respect to this, data regarding the status of host 'non-classical' innate anti viral defense mechanisms like epigenetic regulation are scanty in HBV infection.

Viruses which co-evolved with their hosts, have developed abilities to repress host immunity by epigenetic modulation of immune gene clusters. HBV X protein epigenetically inactivate tumour suppressor protein retinoblastoma (Rb) via the down-regulation of a cellular senescence protein $p16^{INK4a}$ which is considered as a major cause of HBV induced immune evasion and development of HCC [9]. On the other hand, epigenetic modulation by the host through acetylation/methylation of HBV DNA has been identified as a novel mechanism to control viral replication [10,11].

In this process, a conserved group of proteins called DNA (cytosine-5) methyltransferases (DNMTs) help to produce epigenetic changes through target DNA methylation. DNMTs are functionally divided in to 'maintenance' (DNMT 1) and '*de novo*' (DNMT 3a,3b) methyltransferases. In hepatocytes, DNMT1 gene expression has been shown to be upregulated upon exposure to HBV DNA [12]. Methylation of viral DNA was also evident in coincubation experiments of cell lines with HBV [13] which has been corroborated further by documentation of methylated HBV DNA in human tissues [11,14]. Upregulation of DNMTs upon initial exposure of HepG2 and Huh7 cells to HBV has recently been reported as a protective mechanism of host to decrease viral gene expression [15]. Consequence to this, increased CpG methylation of HBV cccDNA has been found to be associated with suppressed replicative activity of the virus in HBV related cirrhosis [16] and impaired virion productivity in HBeAg-positive individuals [17]. Such observations led to the hypothesis that upregulation of DNMTs occur as a control measure to inhibit viral replication by increased methylation of viral DNA and in doing so the host looses control and start methylating its own DNA leading to HCC [15].

Surprisingly, no information is available on the role of DNMT2 in HBV infection which is considered the most evolutionary conserved among other human DNMTs and known to methylate RNA instead of DNA [18]. Furthermore, DNMT2 has been shown to be present in cytoplasmic stress granules, interacts with the proteins involved in processing of RNA and hypothesized as a part of primitive antiviral defense mechanism [19].

The purpose of this study was to analyze the expression profile of PBMC derived DNMT 1,3a,3b and 2 mRNAs from HBV infected subjects belong to different disease categories in relation to their respective serum HBV DNA and surface antigen (qHBsAg) concentrations.

Material and Methods

Patients

One hundred and nine HBsAg positive subjects who attended the Asian Institute of Gastroenterology, Hyderabad, India during the period of November'2010to June'2011 were enrolled in this study. Patients were further categorized on the basis of past history, clinical presentations, anti-HBc IgM/IgG status, imaging data, liver histopathology,Child-Pugh (CP) scores [20] and as per AASLD practice guidelines [21,22] into acute (A; n=33), inactive carriers (IC; n=25), chronic (CHB; n=27), cirrhosis (Cirr; n=11) and hepatocellular carcinoma (HCC; n=13) respectively. None of the subjects had decompensated liver cirrhosis. Patients coinfected with HIV, HAV, HCV, HDV, or HEV were excluded from this study. A group of 41 healthy voluntary blood donors served as controls. Prior informed consents were taken from all the study subjects and the study protocol was approved by the institutional ethics committee.

Serologic and biochemical parameters

The serum HBeAg and anti-HBe status of the subjects were determined by commercial enzyme-linked immunosorbent assay (ELISA) kits (Amar-EASE, Taiwan) as per the manufacturer's instructions. Serum aspartate transferase and alanine transferase levels were ascertained by an automated clinical biochemistry analysis system (Randox,Oceanside, CA).

HBV DNA estimation and genotyping

Viral DNA extracted from sera by the High Pure System Viral Nucleic Acid Kit (Roche Molecular Systems Inc, USA) as per manufacturer's protocol. Amplification and quantitation of extracted HBV DNA was performed by Cobas® TaqMan® 48 Analyzer (Roche Diagnostics, USA) using real time Cobas® TaqMan® HBV test kit (Roche Molecular System, USA) as per manufacturer's instructions. HBV genotypes were determined using an in house nested polymerase chain reaction assay involving type-specific primers to generate respective genotype-specific amplicons [23] followed by direct sequencing.

Quantitation of serum HBsAg

Serum concentration of HBsAg (qHBsAg) was measured by a sandwich ELISA kit (Alpha Diagnostic International, San

Table 1. Baseline characteristics of the patients.

Parameters	Disease categories (n=109)				
	AHB (n=33)	IC (n=25)	CHB (n=27)	Cirrhosis (n=11)	HCC (n=13)
Age (Mean ±SD)	35.3±15.1	34.84±11.19	34.6±12.5	46.7±13.7	48.9±10.8
Sex (M:F)	28:5	22:3	20:7	7:4	13:0
ALT (IU/L – mean ±SD)	928±134.8	31.1±12.5	65.9±21.8	97.8±41.7	90.4±36.0
HBV DNA (log copies/ml – mean ±SD)	5.07±1.77	2.57±1.14	6.48±1.75	4.85±2.34	6.87±2.1
Mean HBs Ag (Log IU/ml – mean ±SD)	3.79±0.71	3.70±0.57	4.02±0.56	4.23±0.31	4.38±0.08

Antonio, TX) having a lower limit of sensitivity of 0.3 ng/mL as per manufacturer's instructions. For convenience, HBsAg concentrations measured in ng/mL were converted to IU/mL, considering 0.15 ng/mL of HBsAg is equivalent to 350 IU/m as described earlier [24,25].

Isolation of total RNA from PBMCs

PBMCs were immediately isolated from EDTA containing whole blood using histopaque-1077 (Sigma chemicals, USA) by recommended protocol. The cells were subjected to RNA isolation by Trizol (LifeTechnologies,USA) method and the extracted RNA was dissolved in diethyl pyrocarbonate (DEPC)-treated water.

Preparation of cDNA

A common cDNA pool was generated by reverse transcription from total RNA using random hexamers and MuLV-H reverse transcriptase (Fermantas Life Sciences, Germany). Before the reverse transcription,1 µg of total RNA was treated with 1 U of deoxyribonuclease (DNase I amplification grade, Gibco-BRL,USA) to remove all the contaminating DNA. The presence of traces of DNA was further excluded by performing control reactions without reverse transcriptase enzyme. RNA was reverse transcribed (60 min at 37°C) with 200 U of MMuLV reverse transcriptase (Fermantas Life Sciences, Germany) in 20 µL volume of 5 × RT buffer (250 mM Tris-HCl pH 8.3, 375 mM KCl,15 mM MgCl2) supplemented with 5 mM dithiothreitol (DDT),0.5 mM deoxynucleoside triphosphates (dNTPs, Fermantas Life Sciences, Germany), 25 U ribonuclease inhibitor (Promega Corporation, Madison, WI, USA) and 200 ng random hexamers (Fermantas Life Sciences, Germany).

Polymerase Chain Reaction (PCR) amplification of candidate genes

After heating (95°C, 1 min) and quick-chilling on ice, an aliquot of 5 µL (0.3 µg) of the cDNA pool was used for PCR amplification in 50 µL of 10 × buffer solution (100 mM Tris-HCl pH 9.3, 500 mM KCl, 1% Triton X-100) containing 0.08 mM dNTPs, forward and reverse primers (40 ng each), 1.5 mM MgCl2 and 2 U of Taq DNA polymerase (Fermantas Life Sciences, Germany). DNMT1 cDNA fragments were amplified by 40 cycles (95°C – 20sec', 55°C – 25sec & 72°C – 25sec per step) using forward and reverse primers (5'-3') d(GGT TCT TCC TCC TGG AGA ATG TC) and d(GTC TGG GCC ACG CCG TAC TG) generating a product of 146bp [15]. DNMT2 cDNA fragments were amplified by 40 cycles (95°C – 20sec', 55°C – 25sec & 72°C – 25sec per step),using up and downstream primers (5'-3') d(ACA GAC TGC AGA GGA TGT GC), and d(TCT TCT CAG GAA ATC CGA ACT C) to yield a product of 167 bp [15]. DNMT3a and 3b cDNA fragments were amplified by 40 cycles each (95°C – 20 sec, 55°C – 25sec & 72°C – 25sec per step)using up and downstream primers (5'-3') d (TAA GCT GGA GCT GCA GGA GT) and d(GGA AAC CAA ATA CCC TTT CCA) for DNMT3a and d (ACC ACC TGC TGA ATT ACT CAC GC ACC ACC TGC TGA ATT ACT CAC GC) and d (GAT GGC ATC AAT CAT CAC TGG ATT) DNMT3b to yield 179 bp and 146 bp amplicons respectively [15]. Beta-actin as house keeping gene was amplified by 30 cycles (94°C, 55°C & 72°C; 1 min per step) using forward and reverse primers (5'-3') d(TCT ACA ATG AGC TGC GTG TG) and d(GGT GAG GAT CTT CAT GAG GT) generating amplicon of 314 bp. Blank reactions without cDNA template were performed in all experiments as negative reaction control. Each amplified product (10 µl) was subjected to 2% agarose gel electrophoresis(100 V,45 min) along with a 100 bp DNA ladder and visualized by UV fluorescence after staining with ethidium bromide.

Upon capturing the gel image by Bio-Capt (Vilber Lourmat, France), the integrated DNA band density was measured by using software Image J 1.42 (Broken Symmetry Software, USA) using the known standard marker as a control with units nanogram per milliliters (ng/ml) and normalized against beta actin used as internal control to define the expression of respective genes by the density of the band.

Statistical analyses

Descriptive statistics (mean, median, standard deviations), Student's *t*-test, and Fisher's Exact tests were performed as and where applicable using SPSS software, version 13.0 (SPSS, Inc., Chicago, IL). A value of $P<0.05$ was considered statistically significant.

Results

All the patients were adults (Mean age ±SD=37.6±13.8 years),and consisted of 90 men and 19 women. The detailed demographic, biochemical and virological characteristics of

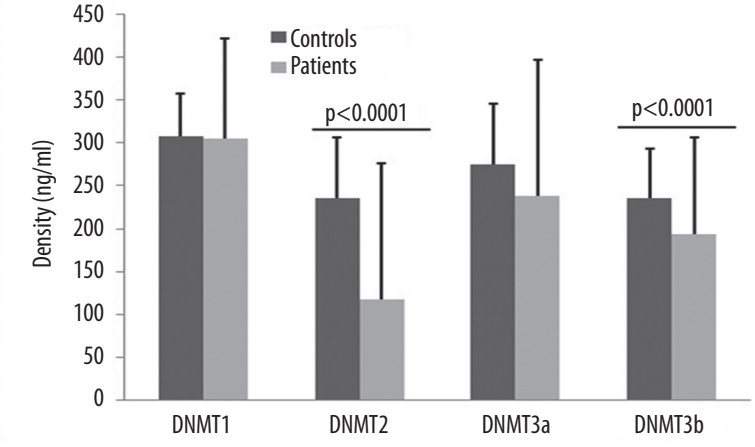

Figure 1. Expression of DNMT2 and 3b genes significantly reduced in patient (n-109) than control (n=41) groups (vertical bar – SD).

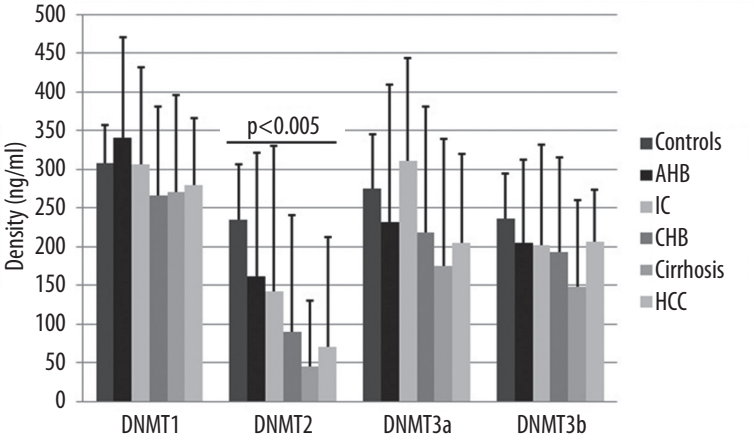

Figure 2. DNMT2 gene expression was significantly suppressed in all categories of HBV infection (AHB – acute hepatitis B; IC – inactive carriers; CHB – chronic hepatitis B; Cirrhosis – cirrhosis of liver; HCC – hepatocellular carcinoma; vertical bar – SD).

109 patients assigned to different disease categories are depicted in Table 1. Control subjects had a mean ±SD age of 29.4±10.1years and consisted of 23 males and 18 females. Of the 109 patients, 84 had genotype D HBV infection,18 had genotype A and 7 were undetermined. Two patients in the AHB group and all 76 patients of other groups were HBeAg negative as well as were positive for anti-HBe antibody.

Expression profile of DNMT 1,3a and 3b genes in control and patients

No significant changes observed in DNMT 1 and 3a expression between the control and patient groups (Figure 1).The observed median value of DNMT1 and 3a in controls and patients were 324.7 (mean ±SD=307.9±49.0), 308.3 (mean ±SD=275.1±70.5), 298 (mean ±SD=305.5±116.0) and 235.1 (mean ±SD=238.2±158.8) ng/ml respectively. However, DNMT 3b gene expression was significantly reduced ($p<0.05$) in patients having median value of 190 (mean ±SD=193.3±112.6) than controls having median value of 212.6 (mean ±SD=235.9±57.9) ng/ml respectively (Figure 1).

Suppression of DNMT 2 gene in HBV patients

For the 109 HBV patients with HBV infection, DNMT 2 gene expression was found to be significantly downregulated ($p<0.0001$) in comparison to the healthy controls (Figure 1). The median expression values(ng/ml) obtained for DNMT 2 in controls and patients were 246.7 (mean ±SD=235.2±70.9) and 0 (mean ±SD=116.9±159.6) respectively (Figure 1).

Comparative expression of DNMT genes in HBV disease categories

DNMT 1 gene showed significant decline ($p=0.04$) only in CHB group having a median value of 226.7 (mean ±SD=265.9±115.4) in respect to controls (median=324.7; mean ±SD=307.9±49.0) without any significant deviation in other disease groups. In comparison to controls (median=308.3; mean ±SD=275.1±70.5). DNMT 3a expression was found to be significantly reduced in cirrhotic (median=144.2; mean ±SD=174.6±164.5; $p=0.004$) and HCC (median=180.9; mean ±SD=204.8±114.8; $p=0.01$) subjects only. DNMT3b expression was significantly down regulated only in cirrhosis group (median=165.9; mean ±SD=147.8;$p=0.0007$) in respect to controls (median=212.6; mean ±SD=235.9±57.9) while no significant changes in the level of expression was observed in any other disease categories.

Strikingly, in comparison to controls (median=246.7; mean ±SD=235.2±70.9), DNMT 2 gene expression was significantly lowered in all the HBV disease categories ($p<0.005$)

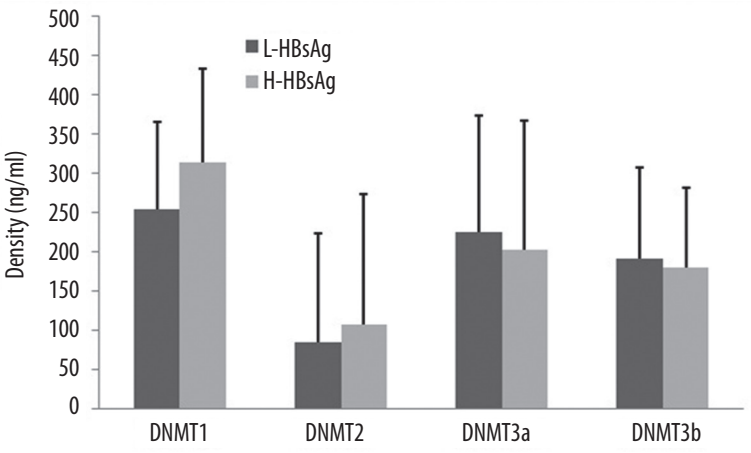

Figure 3. Serum HBsAg concentration had no effect on DNMT gene expression (L-HBsAg – lower concentration[<4 Log Iu/ml] of HBsAg; H-HBsAg – higher concentration[>4 log IU/ml] of HBsAg; vertical bar – SD).

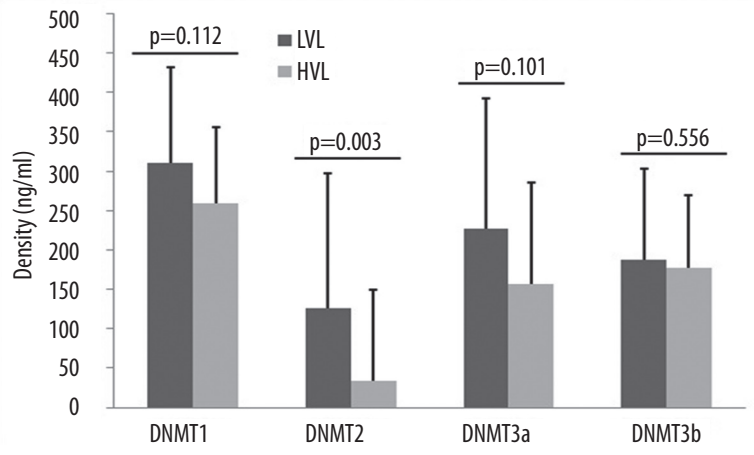

Figure 4. DNMT2 gene expression was significantly suppressed in patients having higher viral load (LVL – low viral load <5 log IU/ml; HVL – high viral load >5 log IU/ml; vertical bar – SD).

which was more pronounced in CHB (median=0; mean ±SD=90.5±150.0; p<0.0001) and cirrhosis (median=0; mean ±SD=44.7±85.7; p<0.0001) groups (Figure 2).

No association of serum HBsAg level and DNMT gene expression

DNMT gene expressions were comparatively analysed in patients having lower level of HBsAg (LHBsAg <4 log IU/ml, n=30) and those having higher HBsAg level (HHBsAg >4 log IU/ml, n=72).No significant change was found in any of the DNMT gene expression between patients having lower or higher HBsAg concentration (Figure 3).

Expression profile of DNMT genes in relation to viral load

Of the 109 patients,104 who had measurable serum HBV DNA were further divided in to two categories based on their viral load in to low viral load (LVL,HBV DNA<5 log copies/ml, n=56) and high viral load (HBV DNA >5 log copies/ml, n=46) groups. No significant differences was observed in the expression profile of DNMT 1,3a and 3b genes between the LVL and HVL groups (Figure 4). In contrast, DNMT 2 gene expression was significantly decreased (p=0.003) in HVL (median=0; mean ±SD=34.5±114.6) than LVL (median=0; mean ±SD=126.2±170.4) patients (Figure 4).

A significant inverse correlation (r=–0.577, p<0.001) was further evident between the increasing viral load and decreased level of DNMT 2 gene expression (Figure 5).

Discussion

DNA methylation operated through various DNMTs is involved in controlling gene transcription, maintaining genome stability and integrity as well as involved in the inactivation of integrated foreign DNA, such as retrotransposons, proviral sequences and other transposable elements [26]. Of members of the DNMT family of proteins, the roles of DNMT1and DNMT3 are relatively well understood. In contrast, our knowledge about DNMT2 is scanty.

Methylation anomalies play a fundamental role in tumorigenesis and immune evasion by the viruses. A strong correlation exists between HBV infection and epigenetic alterations of tumour suppressor genes including $p16^{INK4a}$ [27]. HBx protein has been shown to methylate, and,in turn, downregulate $p16^{INK4a}$ through activated expression of DNMT1 leading to inhibition of senescence and apoptotic proteins via inactivation of tumour suppressor retinoblastoma (Rb) protein [9,28]. According to recent data, DNMTs are upregulated in cells exposed to HBV and are able to methylate HBV DNA, thus can inhibit viral replication

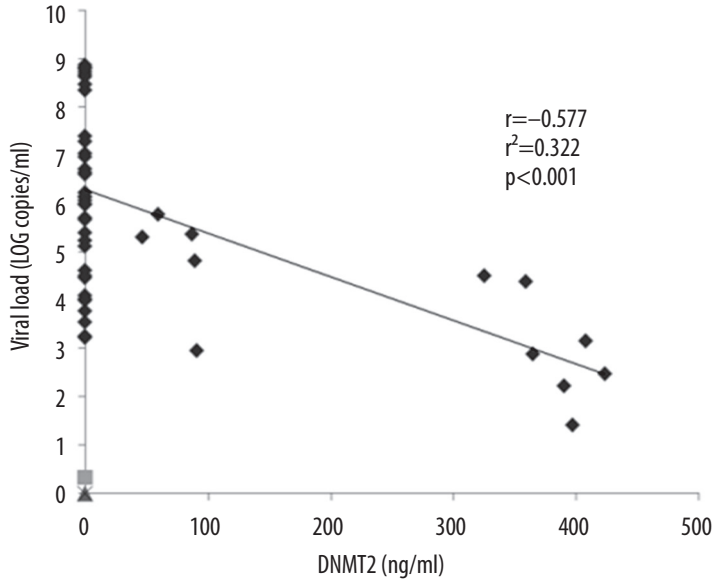

Figure 5. The serum HBV DNA(viral load) level inversely correlated with DNMT2 gene expression in HBV patients.

[15]. Presence of methylated nonintegrated HBV DNA has been demonstrated in human tissues [11,14,17] where methylation of viral DNA reduces viral mRNA and protein production [14]. Also of interest is the finding that HBV replication can cause *de novo* methylation and decreased expression of interleukin-4 (IL-4) [29]. Since IL-4 expression is known to inhibit HBV replication [30], this kind of epigenetic regulation might benefit the virus to survive within the host.

Till now, data regarding the expression of DNMT genes mainly relied on *in vitro* studies and liver tissues obtained from HBV mediated HCC. However, data obtained from previous studies cannot directly address questions of dysregulation of DNMT activities in long-term HBV infection that leads to chronicity. In this respect, while DNMT 1,3a and 3b are mostly studied, DNMT 2 which has involvement in the processing of RNA during stress [19] and thus, is different from the role played by other DNMTs has not been studied in CHB infection.

In our study, while the expression of DNMT1,3a and 3b genes were not markedly altered, DNMT2 gene expression was significantly suppressed in HBV patients irrespective of different disease categories in comparison to controls. Although higher level of HBsAg had no effect, higher viral load significantly (p=0.003) suppressed DNMT2 gene expression where an inverse correlation (p<0.001) was quite evident.

A major obstacle in defining the biological function of DNMT2 in general lies in the lack of phenotypic and molecular data. At present, the role of a RNA methylating enzyme like DNMT 2 in HBV infection is also not known. It has been postulated that DNMT 2 get incorporated in to cytoplasmic stress granules and P-bodies in response to viral infections as a mode of primitive antiviral defense mechanisms under stress and over expression of DNMT2 cause change in expression of several genes, involved in host response to viral infection [19].

RNA-methyltransferases employ different catalytic strategies to methylate viral RNA which is strongly related to other posttranscriptional modifications [31]. Whether such kind of mechanisms are operational in HBV infection or, whether HBV pregenomic RNA serve as a target for DNMT2 remains to be elucidated by further studies. A detailed analysis of DNA methylation in the HBV genome in liver samples of patients at different stages of HCC revealed recurrent hypermethylation in HCC but not in chronic hepatitis tissue [32]. This findings bear some implications to our data on CHB infection where DNMTs as a whole remain not induced. Furthermore, significant suppression of DNMT2 by increased presence of HBV might appear advantageous for the virus to persist in the host.

Although the mechanism of such suppression is not known, further studies identifying the interactive HBV proteins or the candidate micro RNAs regulating DNMT2 must determine whether upregulation of DNMT2 expression has the potential to be considered as an intervening strategy to control HBV replication.

Conclusions

Our data showed that the host DNMT2 gene expression was markedly suppressed in CHB infection and HBV induced cirrhosis of liver having inverse correlation with higher HBV load raising the possibility of its use as a biomarker for HBV induced liver cirrhosis. Considering the association of DNMT2 as a part of antiviral defense mechanism, its sustained suppression by HBV might help viral persistence in the host to develop chronic disease.

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Experimental & Clinical Hepatology

Received: 2012.03.01
Accepted: 2012.03.09
Published: 2012.06.01

Successful pregnancy and delivery in liver cirrhosis – a case report

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B Data Collection
C Statistical Analysis
D Data Interpretation
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Summary

Autoimmune hepatitis (AIH) is a chronic inflammatory disease with a diverse spectrum. AIH is an important cause of liver failure, frequently necessitating orthotopic liver transplantation. Liver transplantation is indicated in patients with chronic autoimmune hepatitis who progressed despite immunosuppressive therapy. AIH is prevalent among young women in their childbearing years. Pregnancy is often desired by these patients. Most women with AIH already have cirrhosis at the time of diagnosis, pregnancy is not expected. Improvement in medical care of cirrhotic women may result in higher conception rates. The risk of maternal and fetal complications is higher than in the general gravid population. The question remains wheter pregnacy is a threat for the patient with AIH or fetus or newborn child. Moreover, the recommendation of the special management in these cirrhotic mothers and their infants during pregnancy should be determined. The present work describes the clinical history of a patient with cirrhosis due to AIH, who had pregnacy and delivered a healthy child. Clinical data regarding the outcome of pregnancy, fetus and mother conditions, maternal complications and mode of delivery have been presented. This patient is especially interesting in that she had an uneventful term of pregnancy and delivery despite liver cirrhosis. She had been doing quite well except for mild nausea. She expressed the wish to deliver this baby. Her general condition was satisfactory. She delivered a healthy male baby in May 2003. The infant weighted 2940 g with an Apgar score of 9/10. The postpartum course was uncomplicated.One month later her transaminases were normal. Prednisone was continued at 20 mg and AZA was restarted at 50 mg. Subsequently prednisone was gradually reduced. At present, she is maintained on prednisone 10 mg. The patient had an uneventful term pregnancy and delivery despite liver cirrhosis. The patient gave birth to a normal male, healthy infant. Liver function was stable during pregnancy and after parturition. Our observation points to a marked immunosuppressive effect of pregnancy on autoimmune liver disease.

In summary, we have demonstrated that succesful termination of pregnancy could be possible in a patient with compensated cirrhosis. Pregnancy leads to attenuation of autoimmune process and the immunosuppressive therapy can be decreased during that period in selected patients. Careful, multidisciplinary monitoring of these complex patients and their infants is required.

Key words: liver cirrhosis • pregnacy

Full-text PDF: http://www.expclinhep.com/fulltxt.php?ICID=997407

Word count: 1354
Tables: –
Figures: –
References: 21

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Bacgkround

Autoimmune hepatitis (AIH) is a chronic inflammatory disease with a diverse spectrum. Although there are different types of autoimmune hepatitis, the clinical presentation might be similar with only slight differences. They are characterized by different autoantibody patterns [1].

The pathogenesis and possible triggering factors remain unknown. Corticosteroids and azathioprine (AZA) are the mainstay of treatment for AIH. This treatment induces a marked improvement in morbidity and mortality [2]. AIH is important cause of liver failure, frequently necessitating orthotopic liver transplantation. Liver transplantation is indicated in patients with chronic autoimmune hepatitis who progressed despite immunosuppressive therapy [3].

AIH is prevalent among young women in their childbearing years. Pregnancy is often desired by these patients. Most women with AIH already have cirrhosis at the time of diagnosis, pregnancy is not expected. Oligo-amenorrhea or anovulation, presumably because of hypothamic-pituitary dysfunction [4], is often one of the presenting symptoms [5].Under adequate immunosuppressive therapy, the disease activity mostly regresses and normal menstruation returns. Higher conception rate and increasing pregnancies among women with cirrhosis is reported [6,7].

The risk of maternal and fetal complications is higher in cirrhotic patients compared with non-cirrhotic pregnant women. Earlier studies of pregnancy indicate an increased risk of prematurity, low birth weight and fetal loss and maternal mortality [6–8], yet recent advances in therapy of chronic liver disease have modified the prognosis of pregnancy in AIH patients [6,7].

Nevertheless, morbidity and mortality remains higher than that of general pregnant women [6]. The question remains wheter pregnacy is a threat for the patient with AIH or fetus or newborn child. What would happen to her and her child ? What could happen to the course of the underlying liver disease? These are the question that each physician would like to be able to answer [8]. Pregnancy will adversely influence the maternal prognosis of cirrhosis [9]. Moreover, erosive esophagitis, a common occurrence in pregnancy, may contribute to hematemesis. Data describing obstetric outcomes among cirrhotic patients are limited.

Cirrhotic women had an increased incidence of 17.24 per cent overall spontaneous abortion rate. However the incidence of congenital malformations does not seem to be enhnced [9,10]. Surviving babies usually show no ill effect or liver disease.

The present work describes the clinical history of a patient with cirrhosis due to AIH, who had pregnacy and delivered a healthy child, clinical data regarding the outcome of pregnancy, fetus and mother conditions, maternal complications and mode of delivery have been presented..

This patient is especially interesting in that she had an uneventful term pregnancy and delivery despite liver cirrhosis. Pregnancy in women with liver cirrhosis is rare, because of reduced ferlility

Case Report

This young patient, was a twenty-two year-old white women, gravida 1, was referred to our department on October 4, 2002 in her 7th week of pregnancy. AIH type 1 with cirrhosis was diagnosed in this patient at age 18 after presenting with arthralgias and hypergammaglobulinemia. At that time, she had an ALT of U/l, and ANA was weakly and SMA strongly positive. Total proteins were reduced with reversal of the albumin/globulin ratio. Prednisone was initiated at 30 mg/day and slowly decreased, whereas azathioprine (AZA) (75 mg) was added. Her transaminases settled down and prednisone was gradually reduced to 10 mg. She was subsequently hospitalized for episode of decompensated liver function. The disease progressed and in April 2001 patient has been evaluated for liver transplantation. The Child-Pugh classification amounts 8 points. Since then, the patient remains under therapy with prednisone 10mg and AZA 50 mg.

One and half year later, she became pregnant. Her ALT 54 was IU/l and serum bilirubin 0,9 mg/dl. She had been doing quite well exept for mild nausea. She expressed the wish to delivere this baby.

Her general condition was satisfactory. Blood pressure was 120/70. On examination, head, eyes, ears, nose, and throat were within normal limits. The thyroid gland was not enlarged. Both lungs fields and the heart were normal to percussion and auscultation. The lower abdomen was protuberant because of the gravid uterus. Neither ascites nor engorgement of superficial veins was noted. The spleen was enlarged and the liver was not palpable. The presence of esophageal and gastric varices was demonstrated by endoscopy.

Laboratory tests included O Rh+blood, hematocrit of 34 per cent, hemoglobin 11.5 g/dl, decreased platelet count of 50,000/mm^3, ALT slightly elevated, normal bilirubin, negative venereal disease test, and Class 1 Papanicolaou smear. Urine was negative for sugar and protein. The AZA was stopped and prednisone was slightly increased to 15 mg. The pregnancy was complicated by transient vaginal bleeding. The patient was managed by bed rest and allylestrenol administration. We observed during pregnancy anemia and thrombocytopenia. Thereafter, that a pregnancy progressed without problems. Liver tests have remained normal. Repeated blood clotting tests during pregnancy showed low prothrombin time, Quick Test 79 per cent. Total proteins were reduced. Creatinine and blood urea remained within normal limits. She experienced no hematemesis during pregnancy. It was ended at nearly full term with a cesarian section. The patient received 4 units of plasma and 2 units of platelets. She delivered a healthy male baby in May 2003. The infant weighted 2940 g with an Apgar score of 9/10. Presently, he is healthy and properly developing child.

The postpartum course was uncomplicated. One month later, her transaminases were normal. Prednisone was continued at 20 mg and AZA was restarted at 50 mg. Subsequently prednisone was gradually reduced. At present, she is maintained on prednisone 10 mg.

Discussion

A total of 117 pregnancies in 92 patients with various types of liver cirrhosis has been reported in literature [9]. Pregnancy is rarely encountered in women with cirrhosis, which is accompanied by severe menstrual irregularities and infertility with disturbance of estrogen metabolism [4,11]. The case reported in this article is, to our knowledge, the first one in Poland. The patient had an uneventful term pregnancy and delivery despite liver cirrhosis. The patient gave birth to a normal male, healthy infant. Liver function was stable during pregnancy and after parturition.

Immunosuppressive therapy was adapted accordingly during pregnancy and after delivery. AZA has been stopped during pregnancy. In our patient liver tests have remained normal despite the fact that AZA has been discontinued. Because not all cases reported in literature improve during pregnancy the dose of corticosteroids was slightly increased. In our patient proactive increase in steroids and AZA dosage immediately after delivery were instituted. It prevented a serious flare-up.

Theoretically, AIH should improve during pregnancy. There are indeed, reported such cases in the literature [11]. However, Buchel et al. have reported in one patient a flare of hepatitis occurred during the first trimester [11]. A similar course of five cases with deterioration during pregnancy have also been reported [12,13]. After delivery, a flare-up of hepatitis was noted [13]. Because flares occur quite often in postpartum period, it seems wise to augment immunosuppressive therapy after delivery.

Our observation points to a marked immunosuppressive effect of pregnancy on autoimmune liver disease. Similar, pregnancy improved the symptoms of rheumatoid arthritis in approximately 75% of patients [14]. Inflammatory bowel diseases (IBD) tend to run a more benign course during pregnancy [15]. Pregnancy leads to a shift of Th1 to Th 2 response [14]. Progesterone promotes Th 2 cells and has anti-inflammatory properties. Estrogens in high doses may inhibit immune activities [17,18]. Pregnancy-induced tolerance might attenuate the disease. Obviously, it is very speculative hypothesis, as long as the pathogenesis of AIH is not clarified.

Although only 1,2% of the absorbed amount of AZA seems to be excreted in the breast milk [19], breast-feeding during treatment with AZA we did not recommend. The agent was reclassified as "probably safe" for neonate receiving breast –feeding [20], when recently has been reported on six women with kidney transplants who were treated with AZA during breast –feeding without side effects in the newborns [21].

Conclusions

In summary, we have demonstrated that successful termination of pregnancy could be possible in a patient with compensated cirrhosis. Pregnancy leads to attenuation of autoimmune process and the immunosuppressive therapy can be decreased during that period in selected patients. Careful monitoring of these complex patients and their infants is required.

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© E&C Hepatology, 2012; 8(1-2): 63-67

Experimental & Clinical Hepatology

Received: 2012.02.02
Accepted: 2012.02.27
Published: 2012.06.01

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
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The influence of HCV molecular variability on antiviral treatment outcome

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Summary

Similarly to other RNA viruses, hepatitis C virus (HCV) displays significant variability within its genome and persists in infected individual as a heterogeneous population of closely-related variants – *quasispecies*. The most divergent sequences include hypervariable region 1 (HVR1) of E2 and NS5A, however, mutations can also be found in relatively stable non-coding regions: 5'UTR or 3'UTR. It is suspected that the viral genetic variability has important clinical consequences, including: altered virulence and tissue tropism, escape from the immune system response, and resistance to antiviral therapies. Therefore, the clinical importance of HCV molecular variability remains under research, especially its impact on the treatment outcome.

The present article summarizes present knowledge about HCV genetic diversity in the context of interferon and ribavirin treatment outcome.

Key words: **HCV • treatment • *quasispecies* • diversity**

Full-text PDF: http://www.expclinhep.com/fulltxt.php?ICID=997408

Word count: 1594
Tables: –
Figures: –
References: 66

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BACKGROUND

The characteristic feature of hepatitis C virus is molecular instability resulting in the formation of diverse viral variants and producing heterogeneous viral population – *quasispecies* [1–4].

Comparative analysis of NS5B sequence revealed six major viral genotypes, each of which was subdivided into several subtypes [5]. Genetic distance between genotypes reaches 30–35%, between subtypes – 20–25% and more subtle differences (1–5%) define *quasispecies* [4].

The complexity and heterogeneity of HCV population is determined by viral *quasispecies,* which arise within the genotypes and subtypes in each replication cycle [1,5–7].

Virologic response rates to treatment were shown to depend on various host (age, weight, sex, race, liver inflammation, stage of fibrosis, some co-morbidities and IL-28 gene polymorphism), and viral factors (HCV genotype and baseline viral load); [8]. To improve sustained virologic response rates, additional pre-treatment and on-treatment predictors are still under investigation. Numerous studies were undertaken to clarify the correlation between HCV genetic variability and treatment outcome.

HCV *QUASISPECIES*

Q*uasispecies* phenomenon is defined as closely-related, but different genetic viral variants present in a single infected individual [9]. Among the pathogens fulfilling the populational criteria of *quasispecies* theory HCV is frequently mentioned [10]. The population size of HCV can dominate the occupied *niche* and its mutation frequency is high enough to generate heterogeneous population of variants with retained basal sequence [11]. Diversity of HCV *quasispecies* population can be considered with respect to its complexity (variants number) and heterogeneity (genetic distance between variants) [12].

Complexity and heterogeneity of HCV population depends on such factors as: mutation frequency, mutation types, interactions between different variants within the population or environmental pressure (immunological response and treatment) [13].

The major causes of HCV high mutation rate are fast replication and errors generated by RNA dependent RNA polymerase, witch lacks a proofreading activity [14]. Experimentally it was assessed that the mutation frequency in one replication cycle may reach 10^{-4}–10^{-5} per nucleotide [6,7]. Considering the average viral load, replication rate, polymerase mutation rate and HCV genome length, daily generation of mutations within viral population reaches theoretically 10^{7}–10^{8} [1].

Nucleotide diversity in HCV genome varies in particular regions. The most variable encodes E2 envelope protein, where diversity between variants may reach 30–50% [15]. In contrast, the 5' and 3' untranslated regions are known to be the most conserved, with diversity lower than 10% [4]. The relatively conserved HCV genes encode viral core, E1 and NS5B [16–18]. They are used to determine genotypes and subtypes of infected patients.

The mutation frequency may differ even within the same E2 gene which contains two highly diverse regions referred as hypervariable regions (HVR-1 and HVR-2). The nucleotide composition within HVR1 sequence between particular viral variants may vary by as much as 80% to 100% [19]. In addition, the frequency of HCV mutations depends mostly on the infection stage and reaches the highest level during its earliest phase [20].

CLINICAL CONSEQUENCES OF *QUASISPECIES* PHENOMENON – TREATMENT OUTCOME

Genetic variability of HCV is likely to have significant clinical implications. These include selection of variants evading immune response, presenting altered cell tropism, virulence and drug resistance [6,21–24].

Standard therapy of HCV infection consists of pegylated IFN alpha and ribavirin. The effectiveness of combined therapy is still unsatisfactory with sustained virological response (SVR) rates ranging from 42% to 46% for genotype 1 and from 76% to 80% for genotypes 2 or 3 [14,25,26].

Many studies attempted to predict the treatment outcome based on pre-treatment and on-treatment viral population heterogeneity and complexity [8,15,19,27–57]. There are two different directions in *quasispecies* research: predictive value of specific nucleotide and amino acid substitutions of HCV and analysis of *quasispecies* population dynamics. To date, studies showed a correlation between the nucleotide sequence variability encoding some viral proteins, such as E2, NS5A or NS5B and drug resistance [15,19,27,29–31,33–35,37,39–41,43–48,52–57].

HVR1 diversity and treatment outcome

Most of tudies analysing HCV *quasispecies* are limited to highly variable viral regions, particularly HVR1. Hypervariable region 1 encodes first 27 amino acids of E2 envelope glycoprotein which is involved in hepatocyte entry and stimulation of the humoral immune response [58–61]. There is no clear evidence that increase in diversity of the viral population would positively or negatively influence the treatment response. However, there is increasing data showing that high pre-treatment complexity of HCV population results in treatment failure, whereas relatively homogeneous baseline HCV population is associated with viral elimination during treatment [5,15,29,43,53]. In recent years it was observed that HVR1 low *quasispecies* complexity before treatment as well as early changes in viral population during treatment may favor sustained therapeutic response [8,15,27,52].

It was also suggested that only early on-treatment HVR1 viral genetic parameters may be credible predictive factors. Formation of homogeneous viral population, preceded by significant reduction in genetic diversity during initial two weeks of therapy led to a final viral clearance, independently of HCV genotype [19,27,44,48]. Analysis of the early HCV changes show extensive complexity reduction of the viral population, especially in patients who exhibited a sustained therapeutic response. Conversely, lack of changes in the HCV population composition favored viral persistence in serum, despite a significant decrease in viral load level [27]. It suggests that viral genetic parameters during the first weeks of

therapy may provide a valuable treatment prognostic information. Monitoring of the viral population during subsequent weeks of treatment would reveal the formation of homogeneous population first of all in sustained responders [27].

NS5A variability and treatment outcome

Previous studies on NS5A also have revealed relationship between *quasispecies* complexity within these region and treatment outcome [30,34,37,40,41,44–47,54–56]. NS5A is a non-structural protein participating in HCV replication complex formation, however its function is still under investigation. It contains several functional domains: interferon sensitivity determining region (ISDR aa2209-2248), PKR binding domain (PKR-BD aa2209-2274), V3 domain (aa2356-2379) and interferon/ribavirin resistance-determing region (IRRDR aa2334-2379) [41].

Quasispecies analysis revealed conflicting results of the relationship between NS5A variability and treatment outcome [36,41,44,45,47,54,55,62]. Some studies showed that high degree of sequence variation within the V3 domain can be a predictive factor of sustained viral response [34,45,47,55]. The contradictory results of Puig-Basagoiti showed that early lower *quasispecies* complexity and diversity correlates with treatment response [47]. Eventually, results of others have revealed no treatment predictive value of V3 domain mutation rate [44,62]. During treatment, sensitive strains usually exhibit decreased variability [47].

The influence of baseline variability of V3 domain on treatment outcome is not clear. Some analyses associated pretreatment low complexity of *quasispecies* with interferon sensitivity [47,54] whereas others reveal no correlation between V3 domain variability and patients viral response [52].

Studies on the PKR-BD nucleotide sequence of HCV have showed association between its genetic variability and treatment response which might be used as a therapeutic prognostic factor [33,44,51]. It suggests that not frequency of mutations but rather their specificity may influence interferon sensitivity [44].

The clinical value of ISDR diversity depends on HCV geographic distribution. The most significant correlation rate between HCV complexity and interferon response revealed studies on Japanese [30,37,40], but not European and north-American population [28,38]. Observed discrepancies can be a result of more variable viral populations and different treatment schedules (higher IFN doses) if compared to Western countries [46,49,51,63]. Further studies confirmed correlation between positive treatment response and increased mutation rate in Western countries, what may indicate its value as therapy prognostic factor [46].

5'UTR variability and treatment outcome

The 5'-untranslated region is the most conserved region in HCV genome. It contains internal ribosome entry site (IRES) located between nucleotides 40-370, which possesses highly stable secondary and tertiary structure composed of four domains with steam-loop structure [64,65]. IRES plays a pivotal role in HCV life cycle and is involved in viral replication. It participates directly in viral protein translation through interactions with ribosomal subunit and cellular translation factors. Any nucleotide changes disturbing its conformation or ribosome binding properties may result in decrease in efficiency of viral protein synthesis [39,66].

It was revealed that 5'UTR *quasispecies* complexity may influence the treatment outcome, but no clear relationship was observed. Most studies showed no treatment predictive value of 5'UTR diversity [36], whereas Zekri reported that low complexity of 5'UTR *quasispecies* population was more frequently reported in responders [57]. Moreover, it was observed that not mutation frequency but rather its localization within IRES domains influence HCV treatment result [39,53]. Mutation located in the base-paring of the IIIb-IIIc domain or downstream to the initiator AUG-4 codon allowed viral elimination and was detected in sustained responders.

Our study revealed a correlation between *quasispecies* composition within the 5'UTR and treatment outcome. We observed that presence of distinct HCV populations in serum and peripheral blood mononuclear cells (PBMC) at baseline is associated with sustained therapeutic response whereas the high 5'UTR sequence stability during treatment favor viral persistence. Furthermore appearance of new variants was observed more frequently in nonresponders (unpublished data).

Other regions

Although correlation between specific nucleotide polymorphisms within the core (C) and NS5B encoding sequence and treatment outcome was reported [8,41], there is no supportive data showing the treatment predictive value of viral population complexity within these regions.

As seen, often contradictory results concerning treatment predictive value of HCV genetic diversity are found in the literature. Discrepancies may be a result of: various study size; PCR-generated artifacts; differences in ethnicity, genotypes, and research methods implemented in various studies.

Conclusions

Although there is no clear evidence whether HCV *quasispecies* genetic behavior can be a credible predictor of treatment outcome, accumulated evidence strongly suggests that changes in the heterogeneity and complexity of viral population before and during treatment may be an important determinant of interferon and ribavirin therapy outcome.

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