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Proinflammatory cytokines (IL-6, IL-18) and apoptotic factors (HP 53, survivin) in patients with alcoholic liver cirrhosis

Andrzej Prystupa[1], Paweł Kiciński[2], Jarosław Sak[3, 4], Andrzej Grzybowski[5, 6], Anna Boguszewska-Czubara[7], Anna Toruń-Jurkowska[8], Jarosław Niedziałek[9], Wojciech Załuska[4]

[1] *Department of Internal Medicine, Medical University of Lublin, Poland*
[2] *Department of Family Medicine, Medical University of Lublin, Lublin, Poland*
[3] *Department of Ethics and Human Philosophy, Medical University of Lublin, Poland*
[4] *Department of Nephrology, Medical University of Lublin, Poland*
[5] *Department of Ophthalmology, Poznań City Hospital, Poznań, Poland*
[6] *Chair of Ophthalmology, University of Warmia and Mazury, Olsztyn, Poland*
[7] *Department of Medical Chemistry, Medical University of Lublin, Lublin, Poland*
[8] *Department of Mathematics and Medical Biostatistics, Medical University of Lublin, Poland*
[9] *Individual Medical Practice, Lublin, Poland*

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Abstract

Background. Apoptosis is involved in the pathogenesis of alcoholic liver cirrhosis. Its development can be triggered by an inflammatory process. In the present study, levels of apoptotic factors – survivin human protein p53 (HP 53) and IL-6, IL-18 were determined according to the stage of liver cirrhosis.

Material and methods. Seventy patients with alcoholic liver cirrhosis, treated in various hospitals of the Lublin region, Poland were included in the study. Serum levels of IL-6, IL-18, HP53 and survivin were determined by the enzyme-linked immunosorbent assay (ELISA) technique.

Results. The serum level of survivin in patients with alcoholic liver cirrhosis was not statistically different from that found in the control group. The level of HP53 was significantly higher in the group of patients with alcoholic liver cirrhosis compared to the control group (16.53±22.69 vs. 0.39±1.31 U/ml; $p<0.001$). Likewise, the level of IL-6 was significantly higher in the group of patients with alcoholic liver cirrhosis compared to the control group (33.83±41.78 vs. 0.88 ± 0.56 pg/ml; $p<0.001$). Moreover, the level of IL-18 was significantly higher in the group of patients with liver cirrhosis compared to the control group (23.96±31.07 vs. 5.3±8.6 pg/ml; $p<0.001$).

Conclusion. In conclusion, increased serum levels of IL-6 and IL-18 were demonstrated in patients with alcoholic liver cirrhosis. Moreover, the liver cirrhosis patients had elevated levels of HP53, which is a marker of apoptosis. Our results did not demonstrate the correlation between the levels of apoptosis markers (survivin, HP53) and the levels of cytokines (IL-6, IL-18) in the blood serum.

Key words

human protein p53, survivin, alcoholic liver cirrhosis, proinflammatory cytokines, apoptosis

INTRODUCTION

Alcohol is one of the most common causes of chronic liver disease [1] in which inflammatory cytokines play an important role. Kupffer cells are essential for the innate immune system, producing various cytokines [2]. Apoptosis is a highly organised and genetically controlled type of cell death, and can occur in response to excessive alcohol consumption. Phagocytosis of apoptotic bodies by hepatic stellate cells may directly stimulate fibrogenesis, [3] which leads to the development of liver cirrhosis [4]. In this study, the levels of apoptotic survivin and HP53 were assessed according to the stage of liver cirrhosis. The levels of IL-6 and IL-18 were also determined according to the stage of liver cirrhosis and levels of apoptotic factors.

Address for correspondence: Andrzej Prystupa, Department of Internal Medicine, Medical University of Lublin, Staszica 16, 20-081 Lublin, Poland
E-mail: aprystup@wp.pl

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MATERIALS AND METHOD

Patients. Patients with alcoholic liver cirrhosis treated in various hospitals of the Lublin region in Poland were randomly enrolled. The study group included 70 patients (47 males and 23 females). All patients presented a history of heavy alcohol consumption in the absence of positivity for serological viral and autoimmune diseases markers. The diagnosis of liver cirrhosis was based on clinical features, laboratory tests, abdominal ultrasound and history of heavy alcohol consumption. The patients with concomitant alcoholic hepatitis were excluded. The stages of cirrhosis were assessed according to the Child-Turcotte-Pugh criteria (Child-Pugh score) as P-Ch A, P-Ch B, P-Ch C. The control group consisted of 18 (10 males and 8 females) healthy individuals without liver disease and who did not abuse alcohol. Both clinical assessment and laboratory tests were used to exclude underlying liver diseases in the control group. Cases and controls were age and gender matched. The study protocol

was approved by the Ethics Committee. All subjects gave their written informed consent for participation in the study. The characteristics of the study population are given in Table 1.

Table 1. Characteristics of patients with alcoholic liver cirrhosis (P-Ch stage A, B, C) and healthy controls

	Controls (n=18)	P-Ch A (n=19)	P-Ch B (n=24)	P-Ch C (n=27)
Age (years)	55.5±8.9	53.7± 12.4	54.1± 11.5	56.7±7.7
Males/Females (n)	10/8	15/4	15/9	17/10
Drinking period (years)	-	13.6±12.4	13.1±11.5	15.2±5.2
Existing medical symptoms				
Ascites (n)	0	3	15	22
Encephalopathy (n)	0	6	12	25
Oesophageal *varices (n)*	0	3	9	24
Bilirubin (mg/dl)	0.59±0.29	4.38±0.96	8.91±3.32	9.27±7.67
Albumin (g/dl)	5.03±0.51	3.21±0.83	2.58±0.63	2.43±0.50
ALT (U/l)	17.9±5.9	103.9±207.6	38.8±27.9	59.2±91.9
AST (IU/l)	18.3±7.1	135.1±219.2	92.9±93.8	121.8±178.2
Urea (mg/dl)	24.4±10.1	32.15±16.58	22.8±18.7	51.25±5.39
Blood platelets (K/µl)	231.3±29.8	189.6±84.6	108±54.7	117.5±61.4
INR	1.26±0.16	1.34±0.32	1.51±0.31	2.01±0.90
MCV (fl)	84.8±3.5	92.6±5.4	90±11.4	97.7±8.2
Na (mmol/l)	140±3.3	133.5±5.9	135.4±3.5	133.9±6.5
K (mmol/l)	4.38±0.39	3.77±0.59	3.86±0.67	3.96±0.68
C-reactive protein (mg/l)	2.5±2.3	13.3±18.1	18.2±17.2	17.1±16.8

Normalranges: bilirubin (0–1.2 mg/dl), albumin (3.5–5.20 g/dl);
ALT – alanineaminotransferase (5–40 U/l);
AST – aspartateaminotransferase (5–40 IU/l), urea (21–43 mg/dl), bloodplatelets (120–400 K/µL), INR (0.86–1.30), MCV (80–94 fl);
K – potassium (3.5–5.1 mmol/l);
Na – sodium (136–145 mmol/l);
C – reactive protein (0–5 mg/l).

Biochemical measurements. The material for the study was peripheral blood obtained from the ulnar vein. Blood samples were collected between 08.00 – 10.00, after an 8–12-hour overnight fast, into clot tubes in the volume of 7 ml. Serum was separated by centrifugation for 10 min at 1,000 rpm, aliquoted and stored frozen at -20 °C until analysis.

The concentration of IL-6, IL-8, HP53 and survivin in serum of patients and controls were measured using the sandwich enzyme immunoassay technique with commercially available quantitative ELISA test kits (Quantikine Elisa, R&D SystemsEurope, Ltd.). Measurements were conducted according to the manufacture's guidelines on a microplate reader (EPOCH; BioTek Instruments, Inc.) at 450 nm. All samples were measured as duplicates and the mean was calculated for data analysis. A calibration curve and a negative control (a blank well without plasma) were run for each test plate.

Statistical analysis. STATISTICA 12 PL was used for data analysis. Continuous variables were expressed as mean ± standard deviation (SD). Before calculations, variables were checked for normality using the Shapiro-Wilk test; the Brown-Forsythe test was applied to test equality of variances. To compare continuous variables between two groups (the control group and the study group), the Mann-Whitney test was used. To compare the results between more than two groups, the Kruskal-Wallis rank test was used, which is a nonparametric equivalent of ANOVA. The Dunn's *post hoc* test was applied for detailed identification of statistically different groups. Correlations among variables were checked with the Spearman's rank correlation test. For all tests, $p<0.05$ was considered as statistically significant.

RESULTS

The level of survivin in the group of patients with alcoholic liver cirrhosis was 28.63±42.06 pg/ml, and did not significantly differ from that in the control group (21.64±21.92 pg/ml) (p=0.74; Tab. 2, Fig. 1). Moreover, no significant differences in this marker were found according to the stage of liver cirrhosis.

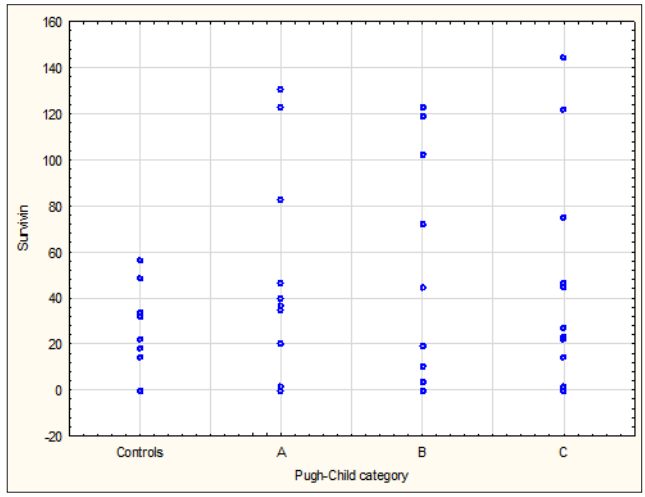

Figure 1. Survivin concentration in cirrhotic patients and controls

The level of HP53 was significantly higher in the alcoholic liver cirrhosis group, compared to the control group (16.53±22.69 vs. 0.39±1.31 U/ml; p<0.001; Fig. 2). Detailed analysis demonstrated significant differences in this marker between patients with P-Ch A cirrhosis (p=0.01; Tab. 2) and the control group, between patients with stage B cirrhosis (p=0.001) and the control group and between patients with stage C liver cirrhosis (p=0.005) and the control group.

Table 2. Levels of survivin, HP53 and the pro-inflammatory cytokines in the control group and in patients with alcoholic liver cirrhosis

Variable	Controls (n=18)	Cirrhosis P-Ch A (n=19)	Cirrhosis P-Ch B (n=24)	Cirrhosis P-Ch C (n=27)	Cirrhosis – total (n=70)
Survivin (pg/ml)	21.64±21.92	32.68±41.42	25.97±43.75	27.43±42.95	28.63±42.06
HP53 (U/ml)	0.39±1.31	7.42±10.62	27.79±31.30	13.11±14.93	16.53±22.69*
IL-6 (pg/ml)	0.88 ± 0.56	17.23±27.68	30.11±24.99	52.08±57.62	33.83±41.78#
IL-18 (pg/ml)	5.3±8.6	18.17±17.73	24.79±30.70	27.94±39.45	23.96±31.07#

*P=0.01 vs. control group; #P<0.001 vs. control group; ^P=0.02 vs. control group

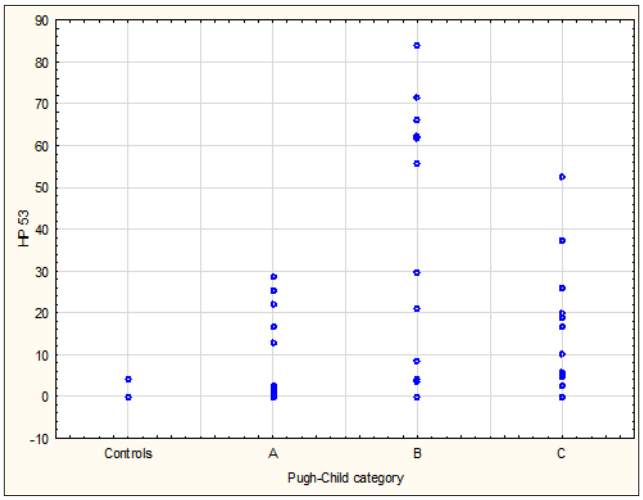

Figure 2. HP53 concentration in cirrhotic patients and controls

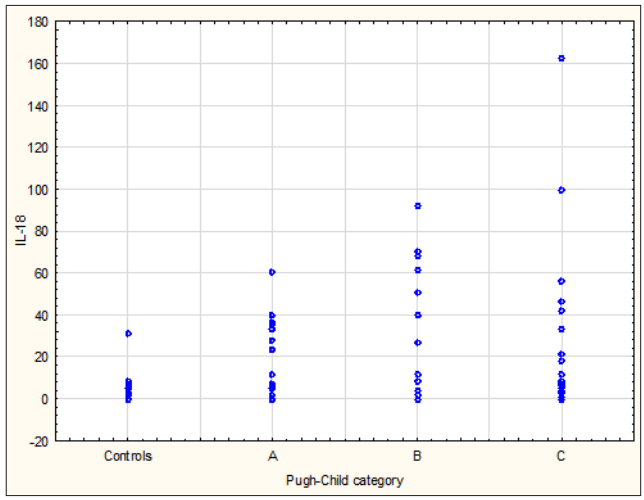

Figure 4. Interleukin-18 concentration in cirrhotic patients and controls

Moreover, the level of IL-6 was significantly higher in the alcoholic liver cirrhosis group, compared to the control group (33.83±41.78 vs. 0.88 ± 0.56pg/ml; p<0.001; Tab. 2, Fig. 3). The level of IL-6 was significantly higher in groups: P-Ch A (p=0.01), P-Ch B (p<0.0001) and P-Ch C (p<0.00001), compared to the control group. Otherwise, there were no significant differences between the various stages of liver cirrhosis (an increasing tendency did not reach the level of statistical significance).

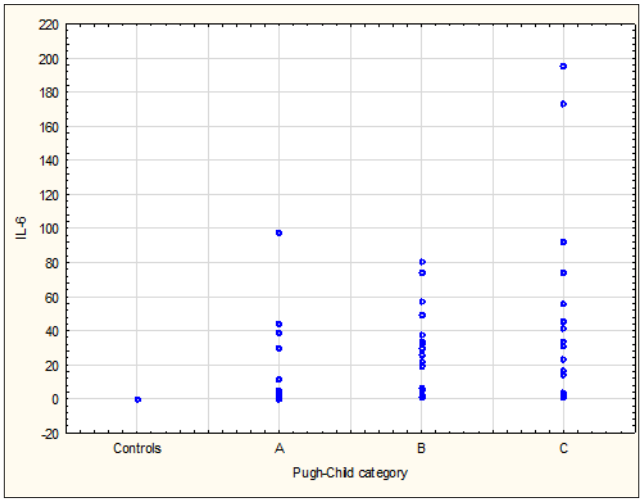

Figure 3. Interleukin-6 concentration in cirrhotic patients and controls

Furthermore, the level of IL-18 was significantly higher in the group with alcoholic liver cirrhosis, as compared to the control group (23.96±31.07 vs. 5.3±8.6 pg/ml; p<0.001). Analysis in the individual subgroups demonstrated significant differences in the level of this marker between the control group and the PCh-C stage subgroup (p=0.02; Tab. 2, Fig. 4).

There were no statistically significant correlations found between the level of survivin versus Hp53 (p=0.18), IL-6 (p=0.67) and Il-18 (p=0.16). Likewise, there were no significant correlations observed between the level of Hp53 versus Il-6 (p=0.48) and Il-18 (p=0.29). Moreover, the levels of IL-6 and IL-18 were not correlated (p=0.58).

Levels of survivin, HP53, IL-6 and IL-18 according to liver cirrhosis complications. The level of IL-6 was significantly higher in liver cirrhosis patients with ascites and otherwise patients with and without ascites (18.23±24.7 vs. 45.83±48.3pg/ml, p=0.02). The presence of hepatic encephalopathy and oesophageal varices was not correlated with the levels of any determined marker. However, a negative correlation was found between the level of HP53 and the platelet count (r=-0.4; p<0.01).

DISCUSSION

Apoptosis has been recognized as a prominent mechanism in the pathogenesis of liver diseases. Apoptosis of hepatic cells occurs in acute and chronic liver diseases. Inflammation, fibrosis and regeneration of the hepatic tissue are associated with apoptotic processes. Apoptosis enables the removal of damaged cells and activates the process of liver fibrosis, which leads to the development of liver cirrhosis [5]. The markers of apoptosis determined in this study were survivin and HP53.

Survivin is an inhibitor of apoptosis protein. Its anti-apoptotic function is associated with the ability to inhibit caspases [6]. An assessment of the level of survivin may have significance in the prognosis of oncological treatment, e.g. multiple myeloma [7], adenoid cystic carcinoma of the lacrimal gland [8], ovarian cancer [9] and osteosarcoma [10]. Peroukides et al. studied the protein expression of survivin in 69 cases of HCC and adjacent liver cirrhosis, and demonstrated increased protein levels and distinct subcellular localization of survivin in HCC and liver cirrhosis [11].

Jia et al. in their study evaluated the diagnostic role of survivin in the serum of patients with HCC. Twenty patients with HCC, 20 patients with liver cirrhosis and 20 healthy volunteers were included in the study. The levels of serum survivin were not found to be significantly higher in patients with HCC and chronic HBV, compared with the control group. No significant differences in survivin levels were observed between the patients with liver cirrhosis and the healthy controls [12]. The findings of the presented study were similar – the level of survivin did not differ in the group with alcoholic liver cirrhosis, compared to the control group, which confirmed the thesis that the level of survivin is not a good marker of liver cirrhosis.

Another marker of apoptosis in alcoholic liver cirrhosis was HP53. The HP53 tumour suppressor protein has a critical

role in regulating apoptosis and G1/S cell cycle arrest [13]. The P53 gene is located on chromosome 17 (17p13.1) and consists of 11 exons (22,000 bp), encoding 2.2 Kb mRNA. The product of gene expression is phosphoprotein HP53 with a molecular weight of 53 kDa. The HP53 protein induces the process of apoptosis only when the damaged DNA has not been effectively repaired [14].

HP53 plays an important role in gastrointestinal cancers [15]. Jackson et al. analysed HP53 mutation in plasma and liver tumour samples, and found a correlation between the occurrence of plasma and tumour mutations of the HP53 gene. The presence of HP53 mutation in the plasma could be indicative of HCC nodules [16]. Zhang et al. have suggested that chrysin (natural flavonoid) may promoted HCC cell apoptosis via activation of the p53/Bcl2/caspase-9 apoptotic signalling pathway [17].

Minouchi et al. have detected the mutations of HP53 gene in 98 tissue specimens of regenerative nodules obtained from liver cirrhosis [18]. Mutations of the HP53 gene were observed more frequently in cirrhotic liver than in livers of patients with chronic hepatitis. HP53 mutations in liver cirrhosis may be a causative factor that leads to hepatocellular carcinoma.

In the presented study, the serum level of HP53 was found to be significantly higher in the group of patients with alcoholic liver cirrhosis, compared to controls. The highest level of HP 53 was observed in patients with stage B alcoholic liver cirrhosis, according to the Child-Pugh classification.

Shahnazari et al. have shown that the concentrations of HP53 in the sera of patients with HBV-related cirrhosis were significantly higher compared to patients with chronic hepatitis B [19]. Attallah has demonstrated that serum and cytoplasmic HP53 protein expressions were more pronounced in patients with HCC and hepatitis C than in patients with liver cirrhosis, and in liver cirrhosis patients compared to liver fibrosis patients [20]. In the currentr study, the serum level of HP53 was analysed in alcoholic liver cirrhosis patients. The Pubmed database lacks studies regarding the levels of HP53 in alcoholic liver cirrhosis patients without confirmed hepatic carcinoma.

In the presented study the levels of 2 interleukins (IL-18 and IL-6) were determined in the serum of patients with alcoholic liver cirrhosis, and their correlation with apoptotic markers (survivin and HP53) was analysed. Interleukin-18 (IL-18) is a proinflammatory cytokine synthesised by Kupffer cells, and activated macrophages that are involved in chronic inflammation, autoimmune diseases and cancers [21]. IL-18 plays a key role in the development of liver injury and participates in the pathogenesis of chronic hepatitis C [22, 23]. IL-18 accelerates the cell apoptosis [24]. In this study, the level of IL-18 was significantly higher in patients with alcoholic liver cirrhosis, compared to the control group. The highest level of IL-18 was found in stage C liver cirrhosis according to the Child–Pugh classification. Similar results were reported Ludwiczek et al. Cirrhotics had higher levels of IL-18 than non-cirrhotics. The IL-18 levels increased with alcoholic liver cirrhosis progression according to the Child–Pugh classification [25].

Another interleukin examined in patients with alcoholic liver cirrhosis was interleukin-6 (IL-6), a major cytokine involved in the inflammatory response which modulates cellular apoptosis in many cells types [26]. Wei et al. demonstrated that serum levels of IL-6 correlate with the severity of rheumatoid arthritis [27]. According to Song et al., the expression of IL-6 is associated with progression of cervical cancer [28]. Deviere et al. have demonstrated elevated levels of IL-6 in patients with alcoholic liver cirrhosis [29], and acccording to Lee et al., the severity of liver cirrhosis is an important factor for the occurrence of increased IL-6 levels [30]. The findings of the presented study are comparable. The level of IL-6 was significantly higher in patients with alcoholic liver cirrhosis, compared to the control group. However, no statistically significant correlations were found between the level of IL-6 and the level of apoptosis markers (survivin and HP53).

Patients with alcoholic liver cirrhosis develop chronic inflammation caused by endotoxaemia. The crucial mechanisms of endotoxaemia are intestinal permeability and bacterial overgrowth in the small intestine [31, 32]. Increased serum levels of cytokines are associated with the chronic inflammatory process.

CONCLUSIONS

Increased serum levels of IL-6 and IL-18 were demonstrated in patients with alcoholic liver cirrhosis. Moreover, the liver cirrhosis patients had elevated levels of HP53, which is a marker of apoptosis. The results obtained did not demonstrate a correlation between the levels of apoptosis markers (survivin, HP53) and the levels of cytokines (Il-18, IL-6) in the blood serum.

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