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ORIGINAL ARTICLE

Differential diagnosis of liver diseases using serum biomarkers

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Summary

Purpose: *Many human blood proteins are synthesised in the liver. Their serum levels may decrease or increase due to liver disorders and some of them serve as useful biomarkers. Determination of serum concentration of different biomarkers has important role in diagnosis of liver diseases and in monitoring the course of disease. In this work 3 serum markers associated with liver disorders were compared. The aim was to assess whether these biomarkers exhibit specific distribution pattern in different types of liver disease: liver neoplasia (primary hepatocellular carcinoma [HC] or metastatic liver disease [MLD] from colon cancer), viral hepatitis C (HCV) and the parasitic infection echinococcosis.*

Methods: *Serum concentrations of α-fetoprotein (AFP), ferritin and insulin-like growth factor I (IGF-I) were determined in patients with liver disease and compared between patient groups and with healthy persons.*

Results: *Serum AFP and ferritin levels exhibited similar pattern of change in patients with liver neoplasia or HCV, and concentrations of these 2 markers were significantly increased compared to the control group ($p < 0.01$ in each case). On the other hand, the concentration of IGF-I was significantly decreased in patients with liver neoplasia or echinococcosis compared to the control group ($p < 0.05$ for both). The concentration of IGF-I was significantly lower and the concentration of ferritin significantly higher in patients with HC than in patients with MLD from colorectal cancer ($p < 0.01$ for both).*

Conclusion: *The results have shown that each hepatic pathology studied exhibited specific profile of the analysed set of biomarkers. Therefore, the simultaneous determination of the 3 mentioned biomarkers may help in differential diagnosis of liver diseases.*

Key words: AFP, echinococcosis, ferritin, liver neoplasia, IGF-I, viral hepatitis

Introduction

Biomarkers are substances that can be detected and whose concentrations changes due to a pathological state in an organism may serve as indicators of a disease [1,2]. Liver is an organ with very intensive metabolic and synthetic functions [3]. Many proteins present in human blood and other tissues are derived from the liver, ranging from the most abundant blood protein albumin to those proteins whose concentrations are low and can be measured in μg/l. Serum concentrations of many of these substances change under different physiological conditions, as well as in liver disorders [4].

Liver pathology is very complex and differential diagnosis is sometimes difficult to achieve [5,6]. Along with biopsy and imaging techniques, serum biomarkers have an important role in managing patients with different liver disorders. Malignancies of the liver are among the most common cancers worldwide, and in some parts of the world the prevalence of HC is particularly high [7-9]. Hepatocarcinogenesis is a multifactorial and multistep process. Many conditions, such as viral hepatitis (hepatitis B or C infection), alcoholic hepatitis or cirrhosis often precede HC, a malignancy with a high mortality rate [3,9,10]. Because of poor therapeutic response, early diagnostic and intensive postoperative monitoring of HC is necessary [3]. Detectable changes in the structure of hepatic tissue are not always a consequence of malignancy, and both malignant and benign pathologies may result in altered blood protein

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pattern. In some cases it is not possible to perform liver biopsy and, on the other hand, if tumor lesions are very small, the result of liver biopsy may be falsely negative [11]. Therefore, besides imaging techniques and biopsy, blood markers have an important role in diagnosis and monitoring of the course of liver disease.

In this work the concentrations of 3 serum biomarkers, AFP, ferritin and IGF-I, were determined in serum samples obtained from patients with different liver diseases.

AFP is widely accepted tumor marker for HC [3, 4,11-13]. Although it lacks the sensitivity needed for screening for HC in the general population, it is used, together with ultrasound, for early detection of HC in high risk populations (such as hepatitis B or hepatitis C carriers, or in regions with the high prevalence of HC) [12,13]. High serum AFP levels are strongly suggestive for HC, but in many patients they remain within the reference range [3,6,13]. Determination of AFP is also useful in postsurgical and therapeutic monitoring of patients with HC [12,13].

Serum ferritin concentration is often found increased in liver neoplasia [14]. Ferritin in neoplasia has different molecular structure compared to normal ferritin. It contains an additional subunit, which is also characteristic for fetal ferritin. This oncofetal ferritin stores less iron than normal tissue ferritin and has different electrophoretic mobility [14,15]. Although serum ferritin is elevated in patients with cancer, it has been shown that tumor cells contain less ferritin than nonmalignant cells [14]. Disturbances in serum ferritin levels in patients with liver malignancies are, thereby, not simply a consequence of an inflammation process or hepatocyte destruction, but more complex mechanisms are involved.

Components of the IGF axis, including ligands IGF-I and IGF-II, their receptors, binding proteins and proteases are enrolled in hepatocarcinogenesis, as well [16,17]. IGFs are synthesized in the liver under the control of growth hormone (GH). In patients with liver neoplasia serum concentration of IGF-I is usually decreased [17].

The aim of this work was to assess the possible interrelationship between the 3 mentioned biomarkers (AFP, ferritin and IGF-I) and 3 different liver diseases: hepatic neoplasia (HC or MLC from colorectal cancer), viral hepatitis and the parasitic infection echinococcosis. The selection of patients was based on the fact that viral hepatitis is one of the etiological factors for HC, and that an echinococcal cyst represents an additional tissue mass, as is frequently found in liver malignancies.

Biomarkers in hepatic neoplasia were further analysed in respect to their etiology (primary HC or MLD from colorectal cancer).

Methods

Samples

Serum samples were collected from healthy persons (n=31, 14 males, 17 females, age range 21-65 years) and patients with liver disease that applied for regular blood testing in INEP during March to October 2008 (n=81, 46 males, 35 females, age range 20-75 years). All samples were collected according to the local ethical principles. Primary diagnosis of a liver disease in patients was established in clinical-medical centers in Belgrade, independently of this investigation. The diagnosis of hepatic disease had been made on the basis of clinical, biochemical, serological and pathohistochemical parameters using standard techniques including ultrasound and computerised tomography.

Control subjects consisted of blood donors and volunteers from among our laboratory personnel. They were screened for parameters that indicate hepatic function (transaminases, gamma-glutamyl transferase, bilirubin, albumin, fibrinogen) and for hepatitis viruses (HBV and HCV). They were also questioned concerning general health, alcohol and medication consumption.

Patients included in this study were divided into 3 groups according to their primary diagnosis: patients with liver neoplasia (n=26, 15 males, 11 females, age range 27-75 years), patients with viral hepatitis infection (n=34, 18 males, 16 females, age range 20-58 years; HCV=24, HBV=10) and patients with echinococcosis (n=21, 12 males, 9 females, age range 28-69 years). Among patients with liver neoplasia, 8 had primary HC and 18 MLD from colorectal cancer. Liver metastasis appeared 1-3 years following resection of colorectal adenocarcinoma. Seven patients lost up to 10% of their weight within a 3-month period. All patients with HCV had liver cirrhosis (Child score B or C), while only half of the patients with HBV had cirrhosis; the rest had only HBs antigen detected in their serum. Since the group of patients with HBV was small and not homogeneous in respect to cirrhotic alteration of the hepatic tissue, it was omitted from further investigation. In patients with echinococcosis antibodies to *E. granulosus* were detected in serum dilutions ranging from 1:80 to 1:640 (result considered positive with antibody titer >1:40).

In 14 patients with liver neoplasia transaminases and/or gamma-glutamyl transferase were elevated above the upper reference limits (transaminases > 40 U/l and gamma-glutamyl transferase > 50 U/l). Activities of these enzymes were elevated in 18 patients with HCV infection and only in 3 patients with echinococcosis.

Blood samples were taken in the morning between 8 and 9 a.m., after 12 h of fasting, sera were separated

within 1 h from venipuncture, and samples were aliquoted and stored at -20º C until assayed.

Determination of AFP, ferritin and IGF-I

Serum concentrations of AFP, ferritin and IGF-I were determined using immunoradiometric assay (IRMA-AFP and IRMA-Ferritin) and radioimmunoassay (RIA-IGF-I), respectively (INEP, Belgrade, Serbia). In order to avoid interassay differences, all measurements for a single parameter were performed in a single run.

Statistical analysis

Statistical analysis was done using statistical software programs Primer of biostatistics (version 5, McGraw-Hill Companies, Dubuque, IA) and MedCalc (version 9.3.9.0. http://www.medcalc.be). Chi-square test was used for testing distribution of obtained data. Normal distribution was found only for IGF-I and results are presented as mean concentration ± standard deviation (X±SD). Values for ferritin and AFP concentrations showed nongaussian distribution, and are presented as median (Me) with central 95% range (between 2.5th and 97.5th percentiles). The difference for a single parameter between all 4 groups was tested using ANOVA (for IGF-I) or Kruskall-Wallis test (for AFP and ferritin). Multiple comparisons for testing the difference between specific groups were performed by Student-Newman-Keuls test (for IGF-I) or Dunn's test (for AFP and ferritin). The differences for AFP and ferritin and the difference for IGF-I between HC and MLD patients were tested using Mann-Whitney and t-test, respectively. A difference with $p < 0.05$ was assumed to be statistically significant.

Results

The results obtained for the measured parameters are presented in Figure 1 and Table 1. AFP concentrations in patients with echinococcosis remained low, as well as in healthy subjects, and they did not exceed 5.0 ng/ml. In patients with HCV or liver neoplasia, AFP serum levels were higher than in healthy persons, they exhibited wide range of distribution, and there was no significant difference between these two groups. The distribution of AFP concentration in different groups is shown in Figure 1A. Serum AFP levels in patients with viral infection or hepatic malignancy were significantly different from the control group (Table 1) and from patients with echinococcosis ($p < 0.01$ and $p < 0.05$, respectively).

Ferritin levels expressed the same pattern of change due to disease as AFP in the analysed groups (Figure 1B). Patients with liver neoplasia or HCV had similar ferritin levels. Ferritin concentrations in these 2 groups of patients were significantly increased compared to the control group (Table 1). Ferritin levels in patients with liver cyst caused by *E. granulosus* were, again, not significantly different from those measured in the control group, but were significantly lower than ferritin concentrations in patients with HCV or liver neoplasia ($p < 0.05$ for both).

Serum IGF-I concentrations were the highest in control subjects, and the lowest in patients with liver neoplasia (Figure 1C). However, there was no difference between control subjects and patients with viral hepatitis. IGF-I concentrations in patients with liver neoplasia and in patients with *E. granulosus* infection were significantly reduced compared to the control group (Table 1) and patients with HCV ($p < 0.05$ for both). In contrast to parallel profile of AFP and ferritin alterations in patients with viral hepatitis and liver tumor, the alterations of IGF-I level were significantly different in these 2 groups ($p< 0.05$).

When the patients with hepatic neoplasia were subdivided into groups (Table 2), the concentration of IGF-I was significantly lower and the concentration of ferritin significantly higher in patients with HC than in patients with MLD ($p < 0.01$ for both). The concentration of AFP did not exhibit statistically significant

Table 1. The concentrations of AFP, ferritin and IGF-I in healthy persons and patients with a liver disease: liver neoplasia, viral hepatitis C (HCV) or echinococcosis

Measured parameters	*Healthy subjects*	*Patients with liver neoplasia*	*Patients with HCV*	*Patients with echinococcosis*
AFP (ng/ml) Me (2.5th-97.5th)	3.1 (2.0-5.0)	4.8* (2.9-631.6)	5.5* (2.9-575.8)	3.6 (2.4-5.0)
Ferritin (ng/ml) Me (2.5th-97.5th)	21.4 (4.6-98.7)	121.4* (3.3-676.8)	100.9* (15.2-405.8)	31.9 (1.6-152.9)
IGF-I (nmol/l) X (SD)	22.5 (5.51)	11.2* (6.07)	20.5 (10.26)	16.1* (5.62)

Me: median, X: mean, SD: standard deviation
*statistically significant difference compared to healthy subjects, $p < 0.05$

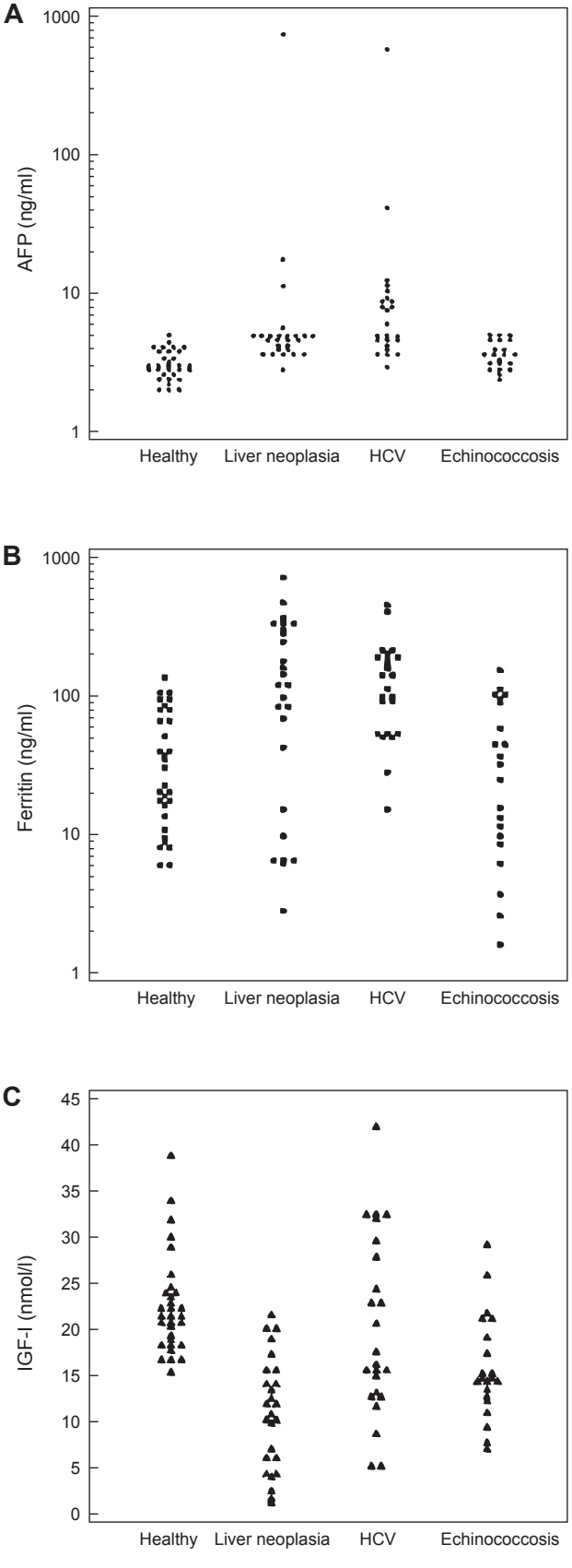

Figure 1. Distribution of AFP **(A)**, ferritin **(B)** and IGF-I **(C)** concentrations in healthy persons and in patients with liver neoplasia, viral hepatitis C (HCV) or echinococcosis. Values for AFP and ferritin concentrations are plotted on logarithmic scale.

Table 2. The concentrations of AFP, ferritin and IGF-I in patients with primary hepatocellular carcinoma (HC) or metastatic liver disease from colorectal cancer (MLD)

Measured parameters	*Patients with HC*	*Patients with MLD*
AFP (ng/ml)	3.8*	4.0*
Me (2.5th-97.5th)	(2.1-648.0)	(2.3-15.9)
Ferritin (ng/ml)	338.7*	24.8§
Me (2.5th-97.5th)	(141.5-671.0)	(1.7-409.8)
IGF-I (nmol/l)	5.3*	13.4*§
X (SD)	(2.83)	(5.35)

Me: median, X: mean, SD: standard deviation
*statistically significant difference compared to healthy subjects, $p < 0.05$
§statistically significant difference between MLD and HC patients, $p < 0.05$

difference between the groups. There was just one extremely high value of AFP (> 700 ng/ml) in HC group (Figure 1), whereas the rest of the results were clustered (< 20 ng/ml). When compared to healthy persons, the only difference in respect to the whole population of patients with hepatic neoplasia was noted for the concentration of ferritin in patients with MLD – it was not significantly different though from the control group.

Discussion

The intention of this work was to try to differentiate 3 liver diseases using serum biomarkers. As said before, liver malignancies are common, with high mortality rate. Many chronic liver diseases precede hepatic neoplasia and early diagnosis of malignancy is very important. Hepatic resection is the most efficient therapeutic procedure for HC, but it is not appropriate for all patients [11]. Another problem are the frequent recurrences after surgical intervention [3,11]. Thus, regular monitoring of patients with chronic liver diseases, which may progress to liver malignancy, as well as patients with HC after hepatic resection or another therapeutic intervention, is necessary. There is no standardized procedure for the surveillance of a hepatic disease, and the complexity of liver pathologies is an additional problem. Besides a need to detect a tumor, it is also necessary to recognize liver diseases other than hepatic malignancy that may cause similar signs. High-risk patients need to be closely followed up. Imaging diagnosis may detect small lesions that are precursors or an early stage of hepatic neoplasia. However, the molecular mechanisms of early hepatocarcinogenesis are far from clear [18]. Liver biopsy allows confirmation of the diagnosis and assessment of prognosis. However, sampling variability is a potential limitation [19]. All

together, in order to achieve the most possible reliability of diagnosis, determination of serum biomarkers is recommended.

Proteomics enables detection and monitoring of a disease by measuring protein concentration and detection of protein isoforms [2,20]. Malignant process can alter protein level in a tissue and, consecutively, in the circulation in two ways: by extensive production of a protein in tumor cells or by intensive destruction of normal cells, which are primary sites of the protein synthesis. Combined detection of different indicators of the pathological process may contribute to higher sensitivity and specificity of a diagnostic procedure. In this work, we combined detection of the routinely used marker AFP with the other two serum proteins synthesized in the liver, ferritin and IGF-I.

AFP is almost an organ specific tumor marker, and high levels of AFP suggest HC [13]. However, AFP concentration is not necessarily increased in patients with HC and, therefore, determination of AFP is not a reliable method for detecting hepatic malignant process. In almost half of the patients with small hepatic tumors, AFP concentration is not increased [21]. In patients with viral hepatitis, a group with particularly high risk for developing HC, AFP may be elevated in the absence of HC [22]. Most patients with viral hepatitis C included in our study had AFP levels below 10 ng/ml, but there was a patient who had extremely high AFP concentration (above 500 ng/ml).

Destruction of liver tissue due to a pathological process influences IGF-I levels in the circulation, suggesting another serum marker of a liver disease [23]. It is known that in chronic liver disorders, cirrhosis in particular, IGF-I levels tend to be lower compared to those in healthy subjects [24]. Reports on IGF-I concentration in patients with chronic hepatitis are, however, contradictory. Okan et al. [25] found that IGF-I levels in patients with chronic hepatitis were increased compared to healthy persons, and they found no relationship with a degree of tissue damage. On the other hand, several authors found significantly decreased IGF-I levels in patients with HBV and/or HCV infection [26-28]. Our results demonstrated similar IGF-I values in the control group and in the group of patients with HCV. Contradicting results may be explained by the fact that IGF-I level in serum is related to the extension of hepatic tissue destruction and the concentration changes during progression of liver fibrosis caused by viral infection [26]. Significant decrease in IGF-I levels was found in patients with echinococcosis or hepatic tumor, in whom, most probably, normal liver tissue was repressed by the presence of a liver cyst or tumor mass [29].

To summarize, patients with liver neoplasia most often had increased ferritin concentrations and sometimes increased AFP levels in blood compared with healthy persons. As a rule, they had low IGF-I concentrations. Similar trends for ferritin and AFP were found in patients with hepatitis C, while IGF-I levels most often remained as in healthy subjects. In patients with echinococcosis, on the other hand, only IGF-I demonstrated reduction compared to the control group. It is worth noting that patients with HC had lower IGF-I and higher ferritin concentrations than patients with MLD. It may be postulated that in a liver with a primary carcinoma the expression of IGF-I is suppressed and the synthesis of ferritin is induced to a greater extent than in a liver metastatically involved. Our results have shown that each hepatic pathology studied exhibited specific profile of the analysed set of biomarkers. Therefore, the simultaneous determination of the 3 mentioned biomarkers may help in differential diagnosis of liver diseases.

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