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BIOMARKERS USAGE IN MINIMALLY INVASIVE DIAGNOSIS OF NONALCOHOLIC STEATOHEPATITIS IN NONALCOHOLIC FATTY LIVER DISEASE PATIENTS

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Abstract

Present study aimed to explore cytokeratin-18, fibroblast growth factor-21 biomarkers usage in minimally invasive diagnosis of non-alcoholic steatohepatitis in patients with non-alcoholic fatty liver disease.

A total of 26 patients (14 males and 12 females) aged from 30 to 60 (mean age 46.36±2.19 years) years with non-alcoholic fatty liver disease in non-alcoholic steatohepatitis stage were enrolled in the study. Control group consisted of 20 healthy volunteers. The average value of the body mass index of examined patients was 27.53 kg/m^2. All participants were diagnosed with non-alcoholic fatty liver disease by ultrasonography and clinical and biochemical data. All patients underwent comprehensive physical examinations, routine clinical and biochemical analyses of blood and urine. Determination of plasma cytokeratin-18 and fibroblast growth factor-21 levels was performed by immunoenzyme method using ELISA kits.

The lipid profile showed a tendency to hyper- and dyslipidemias in patients of the main group. The data indicated a significant increase in liver enzyme activity, the average aspartate aminotransferase level was higher than in the control group for 1.6 times, alanine aminotransferase – for 2.3 times, respectively ($r<0.05$). In patients with non-alcoholic fatty liver disease, non-alcoholic steatohepatitis stage in the levels of cytokeratin-18 and fibroblast growth factor-21 in plasma were significantly increased, correlated with each other ($r=0.806$, $p<0.001$), as well as with aspartate aminotransferase, alanine aminotransferase, total cholesterol and triglycerides, and allowed to establish non-alcoholic steatohepatitis in patients of the main group. Plasma cytokeratin-18 and fibroblast growth factor-21 levels are increased in patients with non-alcoholic steatohepatitis and also associate with atherogenic dyslipidemia and impaired liver enzyme activity. Studying plasma cytokeratin-18 and fibroblast growth factor-21 levels allows establishing the presence of non-alcoholic steatohepatitis in patients with non-alcoholic fatty liver disease.

The findings testify to the possibility of using these biomarkers for minimally invasive screening diagnosis of non-alcoholic steatohepatitis and represent the perspective of further research.

Keywords: *non-alcoholic fatty liver disease, non-alcoholic steatohepatitis, biomarker, minimally invasive, cytokeratin-18, fibroblast growth factor-21.*

Introduction

Early diagnosis of nonalcoholic fatty liver disease is one of the actual problems of modern medicine. The prevalence of the disease, depending on the diagnosis method varies in different epidemiologic studies within 10-35% [*Vernon G et al., 2011*].

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Various aspects of non-alcoholic fatty liver disease diagnosing represents a great clinical interest due to both the incidence rate, in accordance with the growing spread of unhealthy lifestyles, and with the advent of new diagnostic methods to identify the disease [*Scaglioni F et al., 2011*].

Histological variations of non-alcoholic fatty liver disease covering a wide spectrum of manifestations ranging from steatosis to non-alcoholic steatohepatitis, with or without fibrosis, cirrhosis and its complications [*Festi D et al., 2013*]. Lack of

timely treatment of non-alcoholic fatty liver disease can lead to cirrhosis, liver failure and hepatocellular carcinoma. Therefore, it is important to diagnose and treat the early stages of non-alcoholic fatty liver disease [*Miyake T et al., 2013*].

In most cases, fatty liver is latent disease. As for non-alcoholic steatohepatitis, its clinical manifestations are specific, and the signs are often detected during examination for other diseases such as hypertension, obesity, type 2 diabetes, coronary heart disease and others. The most common symptoms are discomfort in the right upper quadrant, fatigue and dyspeptic syndrome. Increased liver enzyme activity indicates the presence of inflammation and cytolysis syndrome, but does not reflect the true extent of liver damage, so has a low diagnostic value [*Maev I et al., 2014*].

Numerous researches and diagnostic tests for non-alcoholic fatty liver disease can detect the stage of disease and liver fibrosis degree. There are different grading scales and indices for minimally invasive and non-invasive diagnosis of non-alcoholic fatty liver disease – FibroMax test complex (FibroTest + ActiTest + SteatoTest + NashTest + AshTest) (France), Fibrometer® fibrous panels (Paris, France), aspartate aminotransferase-to-platelet ratio index (APRI) test, Hepascore® (PathWest, University of Western Australia, Australia). Based on a special mathematical calculation, which includes blood biochemistry and anthropometric data, they can establish the diagnosis, the activity of the inflammatory process, stage of liver fibrosis. Ultrasonography, computed tomography and magnetic resonance imaging are widely used for visualization of non-alcoholic fatty liver disease. These techniques are non-invasive, but none of them has sufficient sensitivity and specificity to distinguish non-alcoholic steatohepatitis and steatosis.

Promising is the use of a new visualizing technique as transient elastography for identifying various non-alcoholic fatty liver disease stages, but more researches are needed for its introduction into clinical practice [*Roĭtberg G et al., 2013*].

Liver biopsy is "the gold standard" for the diagnosis of non-alcoholic steatohepatitis. This procedure is the most accurate method to evaluate the activity of the inflammatory process and stage of liver fibrosis. But, at the same time, hepatic biopsy has some features that do not allow using liver biopsy for the screening of chronic inflammatory liver diseases. This procedure is invasive; there are possibility of complications, diagnostic errors due to the locality of the sample, which can lead to false results and contraindications [*Pearse S et al., 2013*].

To minimize possible risks effective minimally and non-invasive diagnostic strategies are being sought as a screening study and potential alternatives to liver biopsy.

Serum biomarkers that can be used for non-alcoholic fatty liver disease diagnosis are very perspective. The advantages of their usage are minimally invasive, simple and sufficiently accurate method for determining the presence of inflammation and fibrosis of the liver. One of the most promising is a biomarker of hepatocyte apoptosis – cytokeratin-18 (CK-18). A number of studies have confirmed its specificity and accuracy for determining liver steatosis and non-alcoholic steatohepatitis AUROC (area under the receiver operating characteristic curve): 0.83 [*Feldstein A et al., 2009*]; 0.91 [*Younossi Z et al., 2008*]), as well as the ability to use it for non-alcoholic fatty liver disease diagnosis. Cytokeratin-18 may be used both separately and in conjunction with other biomarkers. In a recent study the combination of CK-18 and fibroblast growth factor (FGF)-21 allowed to improve the accuracy of diagnosis of steatosis and non-alcoholic steatohepatitis [*Shen J et al., 2012*].

Traditional risk factors, including cardiovascular diseases are common in patients with non-alcoholic fatty liver disease. Up to 70% of patients with non-alcoholic fatty liver disease have hypertension, about 30-100% – obesity, type 2 diabetes occurs in 10-75% of patients [*Statcenko M et al., 2012; Drapkina O, Ivashkin V, 2014*]. It is proved that the presence of hypertension amplifies or triggers the development of nonalcoholic steatohepatitis. Thus, patients with hypertension in more than 50% cases also have non-alcoholic fatty liver disease without other risk factors for liver disease. It is now believed that hypertension is recognized as an independent predictor of non-alcoholic fatty liver disease [*Fracanzani A et al., 2008*].

Non-alcoholic fatty liver disease is often accompanied by abnormal lipid metabolism, which is considered as an independent risk factor for its development. Changes in visceral type obesity are specially pronounced. Free fatty acids (FFA) due

to intensive lipolysis enter the portal vein and the liver in large quantities. This leads to a decrease of insulin binding and degradation and the development of insulin resistance at the liver level. Suppressant action of insulin inhibits hepatic glucose production and launches systemic hyperinsulinemia, which contributes to the development of peripheral insulin resistance.

Free fatty acids play a special role, caused by its excessive formation, including increased lipolysis in the background of obesity. This leads to excessive accumulation of fat in the uncharacteristic organs and tissues, the formation and development of hepatic steatosis lipotoxicity. Free fatty acids have direct and indirect toxicity of lipid peroxidation. In case of protection mechanisms' damage from FFA direct or oxidative stress-induced mitochondrial injury and hepatocytes apoptosis or necrosis occurs. Inflammation, interacting with the products of lipid peroxidation produced by hepatocytes, becomes an additional source of pro-oxidants in the liver. This provides a cascade of events aimed at forming steatohepatitis [*Gaggini M et al., 2013, Cusi K et al., 2014*].

The atherogenic dyslipidemia is also an independent risk factor, and, according to some researchers, is diagnosed in 20-80% disease cases. Development mechanism is associated with the fact that the synthesis and metabolism of most of the lipoproteins occurs in the liver and is controlled by feedback [*Lazebnyk L, Zvenigorod L, 2009*].

Thus, the features of the development and progression mechanisms of non-alcoholic fatty liver disease and its typical changes represent actual direction for studying various aspects of minimally invasive screening of non-alcoholic fatty liver disease and non-alcoholic steatohepatitis diagnosis, considering concomitant metabolic disorders.

Present study aimed to explore CK-18 and FGF-21 biomarkers usage in minimally invasive diagnosis of non-alcoholic steatohepatitis in patients with non-alcoholic fatty liver disease.

Material and methods

A total of 26 patients (14 males and 12 females) aged from 30 to 60 (mean age 46.36±2.19 years) years with non-alcoholic fatty liver disease in non-alcoholic steatohepatitis stage were enrolled in the study. Control group consisted of 20 healthy volunteers. The average value of the body mass index of examined patients was 27.53 kg/m^2. All participants were diagnosed with non-alcoholic fatty liver disease by ultrasonography and clinical and biochemical data. All patients underwent comprehensive physical examinations, routine clinical and biochemical analyses of blood and urine. Determination of plasma cytokeratin-18 and fibroblast growth factor-21 levels was performed by immunoenzyme method using ELISA kits. Lipid metabolism was assessed by total cholesterol, triglycerides, low-density lipoprotein, very low density lipoproteins and high density lipoproteins. Body mass index was calculated as the weight in kilograms divided by the square of the height in meters. Exclusion criteria were diffuse connective tissue diseases, cancer, acute inflammatory diseases, previous viral hepatitis, toxic (alcohol), medications, congenital metabolic liver disease.

The study was approved by Institutional Bioethics Committee and conforms to the principles outlined in the Declaration of Helsinki (Br Med J, 1964; p. 177) with subsequent additions.

Statistical analysis was performed using "Excel 2010" (Microsoft), "Statistica 7.0 for Windows" (StatSoft Inc.) software packages. Data are presented as mean values and the average error. Analysis of the relationships was carried out with the help of Spearman (r) correlation.

Results and discussion

Lipid profiles and enzyme activity of the liver abnormalities have been identified in non-alcoholic fatty liver disease patients with non-alcoholic steatohepatitis. The results are shown in table 1.

On lipid profile parameters significant differences were detected in patients with non-alcoholic steatohepatitis relatively healthy volunteers. In the main group the average level of total cholesterol was higher than the corresponding index of control group for 30.3%, triglycerides were higher than the control group for 46.0%, atherogenic ratio – 57.6% and low-density lipoprotein for 47.1%.

The data indicated a significant increase in liver enzyme activity in the examined patients compared to the control group. At the same time, the average level of aspartate aminotransferase in blood of main group patients was more than in the control group in 1.6 times, and the mean lev-

Table 1

Indicators of lipid profile and liver enzyme activity in non-alcoholic fatty liver disease patients with non-alcoholic steatohepatitis

Index	Control group (n=20)	Main group (n=25)
Aspartate aminotransferase *(E/l)*	25.6±1.74	42.16±1.32*
Alanine aminotransferase *(E/l)*	22.7±1.7	51.5±1.29*
Total cholesterol *(mmol/l)*	4.25±0.6	5.54±0.3*
Triglycerides *(mmol/l)*	0.8915±0.09	1.3±0.21*
High density lipoproteins *(mmol/l)*	1.4±0.08	1.28±0.14
Atherogenic ratio	1.96	3.09*
Low-density lipoprotein *(mmol/l)*	2.27±0.16	3.34±0.2*

Note: ** – the difference relative to the control group is statistically significant (r<0.05).*

els of alanine aminotransferase in 2.3 times, respectively (r<0.05).

The analysis of CK-18 and FGF-21 levels (Table 2) showed increase of both biomarkers in blood plasma of non-alcoholic steatohepatitis patients relative to the control group.

Table 2

Cytokeratin-18 and fibroblast growth factor-21 in blood plasma of main group patients

Index	Control group (n=20)	Main group (n=26)
Cytokeratin-18 *(E/l)*	92.91±3.64	278.5±2.67*
Fibroblast growth factor-21 *(pg/ml)*	101.96±16.37	203.6±13.01*

Note: ** – the difference relative to the control group is statistically significant (r<0.05).*

In non-alcoholic fatty liver disease patients with non-alcoholic steatohepatitis CK-18 levels were elevated compared to the control group for 3 times and amounted to 278.5±2.67 *U/L*. The levels of FGF-21 also demonstrated a significant increase in non-alcoholic steatohepatitis patients. The result of the main group was 203.6±13.01 *pg/ml*, which exceeded the index of the control group for 2 times.

Revealed correlations between the studied markers, indicators of liver enzyme activity and lipid profile of the main group are presented in table 3.

Analysis of the relationship using Spearman correlation coefficient showed a strong direct link between CK-18 and FGF-21 (r=0.806 p<0.001). Also, the relationship has been established between CK-18 and aspartate aminotransferase (r=0.58 p<0.001), alanine aminotransferase (r=0.68 p<0.001), total cholesterol (r=0.41 p<0.001) and triglycerides (r=0 27 p<0.004). The ratio of FGF-21 with specified indicators demonstrated direct links with alanine aminotransferase (r=0.61 p<0.001), aspartate aminotransferase (r=0.49 p<0.001), total cholesterol (r=0.39 p<0.001) and triglycerides (r=0.21 p<0.023).

Table 3

Analysis of correlations between cytokeratin-18 and fibroblast growth factor-21, and some metabolic parameters in non-alcoholic fatty liver disease patients in non-alcoholic steatohepatitis stage

	Aspartate aminotransferase		Alanine aminotransferase		Total cholesterol		Triglycerides	
	r	*p*	r	*p*	r	*p*	r	*p*
Cytokeratin-18	r =0.58	<0.001	r=0.68	<0.001	r=0.41	<0.001	r=0.27	<0.004
Fibroblast growth factor-21	r=0.49	<0.001	r=0.61	<0.001	r=0.39	<0.001	r=0.21	<0.023

Note: *p<0.05 – statistically significant difference.*

Similar data were obtained and published by H. Li and co-authors, where elevated levels of FGF-21 and their correlation with the level of triglycerides were found in blood plasma non-alcoholic fatty liver disease patients [*Li H et al., 2013*]. Fibroblast growth factor-21 is adipokine, which is produced in humans mainly in the liver and adipose tissues, and acts by metabolic signaling cascade [*Iglesias P et al., 2012*]. Some authors also investigated the increased FGF-21 levels in patients and concluded that this may serve as a biomarker for noninvasive diagnosis of non-alcoholic steatohepatitis [*Dushay J et al., 2010; Morris-Stiff G, Feldstein A, 2010*]. In the study of J. Chen and co-authors increased level of CK-18 apoptosis marker was found in non-alcoholic fatty liver disease patients, especially with non-alcoholic steatohepatitis. The study of correlations in this research showed a direct correlation between marker of apoptosis and alanine aminotransferase [*Chen J et al., 2014*].

Conclusion

The liver metabolic disorder progression in patients with non-alcoholic steatohepatitis is accompanied by increased plasma levels of cytokeratin-18 and fibroblast growth factor-21 and is associated with atherogenic dyslipidemia and impaired liver enzyme activity.

Study of CK-18 and FGF-21 plasma levels allows establishing the presence of non-alcoholic steatohepatitis in patients with non-alcoholic fatty liver disease. The findings testify to the possibility of using these biomarkers for minimally invasive screening diagnosis of non-alcoholic steatohepatitis and represent the perspective of further research.

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