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MICRORNA-122: PROGRESSION TO THE STAGE OF SEVERE FIBROSIS

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Summary
This article provides information on the role of miRNAs in the progression of chronic diffuse diffuse liver diseases from the stage of chronic viral hepatitis to severe fibrosis. The study of microRNAs is important not only for a fundamental understanding of the mechanisms of intracellular regulation, but also has a high practical value as non-invasive biomarkers for the early diagnosis of liver fibrosis.

Keywords: chronic viral hepatitis, microRNA, liver fibrosis

Relevance

Chronic hepatitis is a comprehensive problem of diseases of the digestive system. According to the WHO, more than 170 million people suffer from chronic hepatitis C, and there are more than 2 billion people with signs of current or completed viral hepatitis B infection. High mortality is due to complications of chronic liver disease (CKD), such as decompensated liver failure, hepatocellular carcinoma (HCC). About 1 million patients die every year [4, p. 28; 31, 6, p. 51; 56, 10, p. 31]. In 2016, based on the studies of a large international study, the following conclusions were made,

namely, mortality from chronic viral hepatitis is comparable to tuberculosis, malaria and HIV. A date has been set for World Hepatitis Day on 28 July.

The main causative factors of chronic liver diseases are viral hepatitis B and C, alcoholism, steatohepatitis, etc. The World Health Organization is committed to the global elimination of both hepatitis B virus (HBV) and hepatitis C virus (HCV) by 2030.

MicroRNAs, evolutionarily highly conserved, small (18–25 ribonucleotides) noncoding RNAs, are involved in the regulation of almost all cell functions [2,10,13]. They are currently emerging as a new class of biomarkers. MiRNA dysregulation plays a key role in the pathogenesis of various diseases. A specific feature of miRNAs is their stability in the bloodstream [9, 12]. MicroRNA-122 is involved in the regulation of the expression of genes involved in carbohydrate, lipid and iron metabolism in the body. The data of experimental studies of miR-122 also showed participation in the pathogenesis of hepatitis C, thereby ensuring the life cycle of the virus in the cell. The transition of the study of miR-122 from fundamental research to clinical practice seems promising in the development of personalized medicine. Many international studies have shown their importance as innovative markers in various pathological conditions of the body [1,4,7]. The advantages of microRNAs in this regard include versatility of detection, relative stability, and high sensitivity during sample storage. To date, more than 4000 miRNAs have been described [3,5,6]. Circulating miRNAs in serum are stable and protected from disappearance in body fluids, which makes them universal biomarkers in many diseases [9,10]. In the liver, miRNA-122 accounts for approximately 70% of all miRNAs and is important for the functional state of the hepatocyte, while other organs express a much smaller amount of this miRNA. MicroRNA -122 regulates many genes in the liver that control the cell cycle, differentiation, proliferation, and apoptosis [4,7]. Loss of miRNA-122 in the liver leads to dedifferentiation of the liver with a malignant phenotype. Early diagnosis, determination of laboratory and genetic markers of fibrosis, development and implementation of minimally invasive, effective and affordable methods for assessing the severity and rate of progression of AF seem to be a very important task for modern practical hepatology [8,11].

According to the literature, with the development of liver diseases, in particular acute and chronic hepatitis, as well as their complications such as cirrhosis of the liver and HCC, the functioning of microRNA-122 is disrupted. Therefore, according to modern concepts, a change in the expression level of miRNA-122 can be a prognostic marker of the pathological state of the liver.

The aim of the study is to optimize the methods of non-invasive diagnosis of liver fibrosis in patients with chronic viral hepatitis.

To study the role of microRNA-122 in the study, 32 patients were selected from the total number of examined patients: 17 (53.1%) of them with a diagnosis of chronic viral hepatitis made up subgroup 1 and 15 (46.9%) with a diagnosis of compensated cirrhosis of the liver, subgroup 2 , from among those hospitalized for inpatient treatment in the department of hepatology of the Scientific Research Institute of Epidemiology, Microbiology and Infectious Diseases of the Republic of Uzbekistan. Genetic studies were carried out on the basis of the laboratory of molecular medicine and cellular technologies of the Republican Scientific and Practical Center of Hematology of the Republic of Uzbekistan.

We isolated 10 healthy volunteers from the control group. In group 1 of 17 patients, the age ranged from 30 to 58 years (mean age 41.5±6.8 years), and in group 2 of 15 patients, whose age ranged from 31 to 60 years (mean age 31.5 ±6.8 years).

Material and Research Methods

Material and methods: to study the role of microRNA-122, 32 patients were selected from the total number of examined patients: 17 (53.1%) of them with a diagnosis of chronic viral hepatitis made up 1 subgroup and 15 (46.9%) with a diagnosis of compensated cirrhosis of the liver 2 subgroup.

Peripheral blood samples were collected in tubes and citrate was added as an anticoagulant. Each sample was centrifuged at 3000 g for 7 min at 4°C to completely remove cellular components, and the supernatant was stored at –70°C. For genotyping and determining the load of HCV, the PCR method was used. HCV genotypes 1a, 1b and 3 were found in patients with CHC. RNA isolation and reverse transcription. RNA was extracted from blood plasma samples using a TRIZOL reagent (Invitrogen, United States). Briefly: 1 ml of TRIZOL reagent was added to 250 µl of blood plasma supernatant, according to the manufacturer's recommendations. In order to improve the precipitation of small RNAs, the supernatant obtained from chloroform extraction was mixed with an equal volume of anhydrous ethanol (Merk) and left overnight at –80°C. The formed precipitate was separated by centrifugation at 12000 g for 30 min at 4°C and washed with 70% ethanol cooled to 0°C. After dissolution in RNase-free water, the purity of the isolated RNA was assessed spectrophotometrically: by the ratio of the optical density of the solution (A) at wavelengths of 260/280 nm using the ND2000 system (NanoDrop Technologies, USA). The acceptable degree of purification corresponded to the value A260/280 ≈ 1.8–2.0. To obtain cDNA, an RNA sample was first treated with DNase, and then

polyadenylation and reverse transcription were performed using, respectively, poly(A) polymerase and specific primers. The resulting product was diluted 20 times with deionized water containing neither DNases nor RNases and stored at –80°C. The level of miRNA-122 expression in blood plasma was measured using reverse transcription PCR in accordance with the TaqMan miRNA analysis protocol. Total RNA was extracted with TRIZOL reagent (Invitrogen, CA, USA) according to the manufacturer's instructions. The RNA concentration was measured using a NanoDrop ND2000 spectrophotometer (NanoDrop Technologies, USA). Reverse transcription was performed using the miScript Reverse Transcription Kit (QIAGEN, Germany). The expression level of mature miRNA-122 was studied using the miScript SYBR Green PCR Kit (QIAGEN, Germany) according to the manufacturer's instructions. The miRNA level is calculated by the formula (2 Ct*100), normalized to U6 snRNA and presented in conventional units. Amplification was carried out on Rotor Gene 6000 and Rotor Gene Q devices (Qiagen, Germany). The obtained data are analyzed using software in the form of graphs. The obtained digital data are processed using the Microsoft Office 2007 and Statistica 6 software package (StatSoft Inc., USA). (5% is taken as the critical value of the significance level. Analysis of the correspondence of the distribution to the law of normal distribution of signs was performed using the Shapiro-Wilk test (in the case of miRNA ($p=0.0152$), miRNA $p<0.05$, the distribution of the sign differs from normal. Considering that W then the probability of differences in samples is carried out by a non-parametric method).

Isolation of total RNA from blood plasma containing a fraction of mature miRNAs was performed using the Ribo-prep-100 kit (Central Research Institute of Epidemiology, Rospotrebnadzor, Russia) in accordance with the manufacturer's protocol. The isolated RNA was quantified on a Qubit-4™ fluorimeter (Invitrogen, USA) using a Qubit microRNA Assay Kit-100 reagent kit for the quantitative determination of microRNAs (5–100 ng) (Thermo Fisher Scientific, USA) according to the manufacturer's protocol. The RNA concentration in the sample was calculated automatically using a calibration curve. The yield of "pure" RNA ranged from 0.5 to 30 μg/mL. The reverse transcription reaction was carried out using the MMLV RT kit (Evrogen, Russia) for the synthesis of cDNA on an RNA template and “stem-loop” primers (20 μM) for certain miRNA-hsa-miR-122, U6snRNA (TaqMan microRNA Assays, Thermo Fisher Scientific, USA). The average value of the relative level of miR-122 was determined after the polymerase chain reaction in duplicate for each sample in real time on a Rotor-Gene Q instrument (Qiagen, Germany).

The results ΔCT = miR122 Ct - U6 Ct were normalized in relation to the group of healthy individuals. The results of the determination of miR-122 in groups were

presented in relative units as the arithmetic mean value and the error of the arithmetic mean. The sample size was not previously calculated. The Mann-Whitney test (U) and chi-square test ($\chi 2$) were used to analyze differences between groups.

Results and Discussion

In the course of the study, an analysis was made of the dependence of miRNA 122 expression on sex and age. The correlation coefficient between the expression level of microRNA-122 and the duration of the pathological process was calculated (r=-0.36). The duration of the disease in patients of group 1 averaged 5.7±3.2 years in men and 3.7±3.3 years in women. The duration of the disease in patients of the 2nd group was clearly different and averaged 6.8±1.8 years for men and 3.8±2.2 years for women. 4 ranges were distinguished: low 0.001 - 0.14, medium 0.15 -1.05, high 1.05 - 12.88 and the highest -> 12.89. Analysis of miR-122 expression level showed that patients with low miR-122 levels in blood serum had the longest period of the disease.

Differences in the frequency of occurrence of patients in the 1st and 2nd subgroups of patients in the miR-122 range of 0.001–0.14 was 5.9% versus 26.7%, respectively. The calculated chance of detection and risk of complications in this range is 4.5 (95% CI 0.57 - 36.22) and 5.8 (95% CI 0.57 - 59.31), respectively. However, despite the high rates of OR=5.8 and RR=4.5, such a difference was not statistically significant ($\chi 2=1.3$, $P>0.3$).

In the range of 0.15-1.05, the frequency of miR-122 occurrence in patients in subgroups 1 and 2 was 11.8% versus 66.7%, respectively. The calculated chance of detection and risk of complications in this range is 5.7 (95% CI 1.46-21.86) and 15 (95%, CI 2.4-93.0), respectively, High scores OR=15 and RR=5.7, the difference was statistically significant ($\chi 2=8.0$, $P<0.001$), In this range, the risk of developing liver cirrhosis increases, and we clearly see this.

Differences in the frequency of occurrence of patients in the 1st and 2nd subgroups of patients in the range of 1.05–12.88 was 52.9% versus 6.7%, respectively. The calculated chance of detection and risk of complications in this range is 7.9 (95% CI 1.13-55.58) and 15.7 (95% CI 1.67-148.1), respectively. However, despite the high OR=15.7 and RR=7.9, the difference was statistically significant ($\chi 2=5.9$, $P<0.01$). In the third range, RR increases by almost 8 times, the risk of detection, i.e., OR increases by 15.7 times. In this case, a significant association was found between the expression level of miRNA-122 and stage 4 fibrosis according to ultrasound data.

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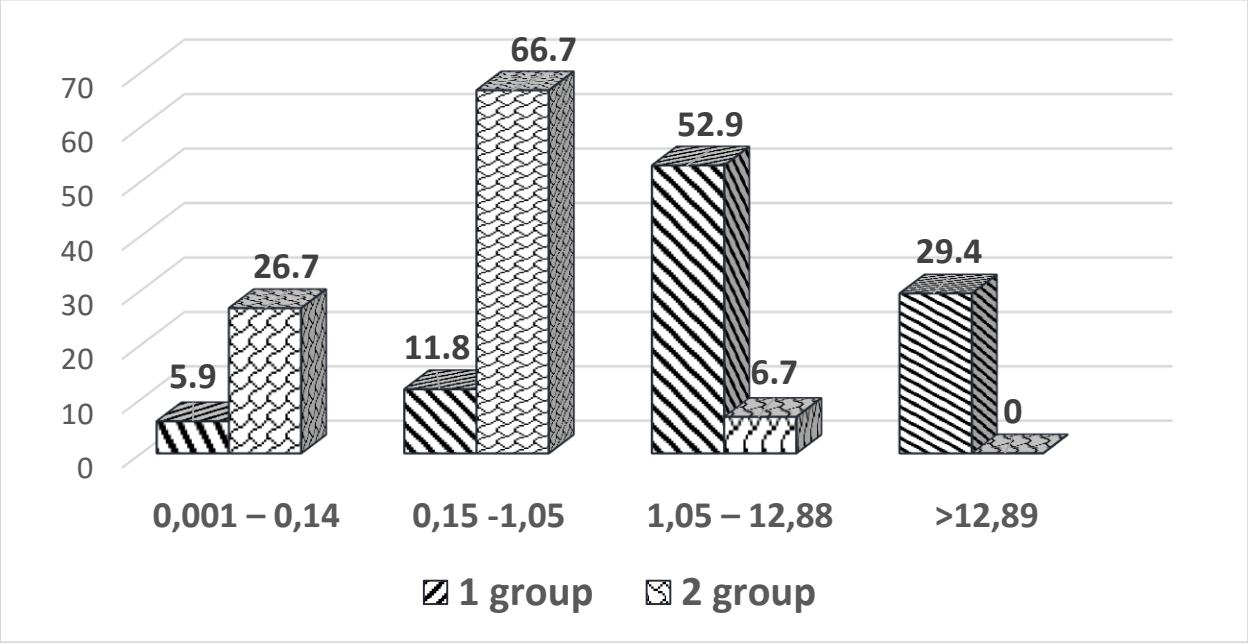

Picture 5.1. Expression level of microRNA-122

Differences in the incidence of patients in groups 1 and 2 of patients in the miR-122>12.89 range was 29.4% versus 0%, respectively. The calculated chance of detection and risk of complications in this range is 4.7 (95% CI 0.614 - 36.03) and 6.2 (95% CI0.64 – 60.93), respectively. However, despite the high rates of OR=6.2 and RR=4.7, such a difference turned out to be statistically insignificant ($\chi 2=1.6$, $P<0.05$), since no patients were observed in this range in group 2. Perhaps with an increase in the number of patients, the differences would be more pronounced. Therefore, a decrease in the level of miRNA-122 expression in the blood plasma reflects the risk of progression of liver fibrosis in humans.

The problem of CVH is considered as a multifactorial nosology. Since not only biochemical parameters play a role in the progression of liver fibrosis, but also violations of molecular genetic aspects. The results of our research are confirmed in a number of foreign studies of recent years, and our data also testify to this. But this study is different from other foreign studies in that we determined the level of miRNA expression in the population of Uzbekistan. Since the literature confirms the difference in miRNA-122 expression levels depending on ethnicity. The results of the study show that the level of microRNA-122, specific for hepatocytes, is significantly reduced in patients with a pronounced stage of fibrosis. Serum miRNA-122 is a new potential parameter of liver function and prognostic parameter in patients with liver cirrhosis.

Conclusions

1. Therefore, a decrease in the level of miRNA-122 expression in the blood plasma is undoubtedly one of the pathophysiological links in the development of fibrosis and its complications in patients with CVH.
2. Modern medicine regards CKD as a multifactorial pathology, which, in addition to etiological factors, is based on complex violations of biochemical and molecular genetic processes.
3. The severity of the development of fibrosis in the examined patients was confirmed by the presence of changes in the indices of cytolysis, protein-synthesizing function of the liver, cholestasis disorders, as well as the results of ultrasound.
4. The results of our study show that the level of hepatocyte-specific microRNA -122 is reduced in patients. Serum miRNA-122 is a new potential parameter of liver function and a predictor of the risk of complications.

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