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THE LEVEL OF EXPRESSION OF MIR-196A IN PATIENTS WITH CHRONIC VIRAL HEPATITIS C WITH THE FIRST GENOTYPE OF HCV ACCORDING TO PREVIOUS EXPERIENCE OF ANTIVIRAL THERAPY

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Ключевые слова: *хронический вирусный гепатит С, микроРНК-196а, противовирусная терапия*

Abstract. The level of expression of miR-196a in patients with chronic viral hepatitis C with the first genotype of HCV according to previous experience of antiviral therapy. Shostakovych-Koretskaya L.R., Shevchenko-Makarenko O.P., Lapikova-Bryhinska T.Yu. *The authors present the study of the level of expression of miR-196a in 74 patients with chronic viral hepatitis C with the 1st genotype of HCV, based on previous experience in patients with antiviral therapy regimens containing interferon. The patients were divided into two groups, depending on the previous experience of antiviral therapy with circuits containing interferon – 21 patients who failed after antiviral therapy regimens containing interferon (group 1) and the comparison group (group 2) – 53 naive patients. To study the level of expression of miR-196a (miR-196a), a two-stage study according to the manufacturer's protocol was used. First, total RNA was isolated from the plasma by the method of phenol-chloroform extraction. Futher reverse transcription was performed using a kit for reverse transcription of miR TaqMan® (Applied Biosystems, USA), specific loop primers to achieve mature miRNA, snRNA U6 (as an endogenous control gene), and 10 ng of total RNA. Real-time quantitative PCR was performed using TaqMan® miRNA analysis. In order to optimize the prediction of response to antiviral therapy and the use of optimal treatment regimens for problem patients with treatment failure regimens containing interferon, analysis using ROC curves was used. The average level of expression of miR-196a in patients with chronic hepatitis C was evaluated. In the 1st group of patients it was 0.011 (IQR: 0.002; 0.310) and in the comparison group – 0.346 (IQR: 0.054; 1.239) at p=0.012 by U criterion. The conducted ROC analysis showed that the studied miR-196a could differentiate patients with chronic viral hepatitis C with HCV genotype 1, depending on previous experience of antiviral therapy, namely, patients with treatment failure regimens containing interferons and naive. AUC=0.688 (95% CI 0.570-0.791; p=0.017), J=0.40, Se=57.14%, Sp=83.02%. It gives additional opportunities for correction of therapeutic tactics. Therefore, the level of expression of miR-196a (<-1,75) may be an additional biomarker in the pathogenesis of HCV-infection, which can then be used in the monitoring and treatment of patients. Low expression of miR-196a may be the basis for prescribing more effective direct-acting antiviral therapy to patients and will allow personalizing therapeutic tactics in patients with chronic viral hepatitis C.*

Реферат. Рівень експресії мікроРНК-196а у хворих на хронічний вірусний гепатит С з першим генотипом HCV, залежно від попереднього досвіду противірусної терапії. Шостакович-Корецька Л.Р., Шевченко-Макаренко О.П., Лапикова-Бригинська Т.Ю. *Авторами представлено вивчення рівня експресії мікроРНК-196а у 74 хворих на хронічний вірусний гепатит С з 1-м генотипом HCV. Хворих було розподілено на дві групи залежно від попереднього досвіду противірусної терапії схемами, що містять інтерферон – 21 хворий, які мали невдачу після проведення противірусної терапії (група 1) та група порівняння (група 2) – 53 наївних*

пацієнти. Для вивчення рівня експресії мікроРНК-196а (miR-196a) використовувалося двоетапне дослідження згідно з протоколом виробника. Спочатку тотальну РНК виділяли з плазми крові методом фенол-хлороформної екстракції. Потім виконували зворотну транскрипцію з використанням набору для зворотної транскрипції мікроРНК TaqMan® (Applied Biosystems, США), специфічних петльових праймерів до зрілої мікроРНК-196а та snRNA U6 (як ендогенного контрольного гена) і 10 нг загальної РНК. Кількісну ПЛР у реальному часі проводили з використанням аналізу мікроРНК TaqMan®. З метою оптимізації прогнозування відповіді на ПВТ та призначення оптимальних схем терапії для проблемних хворих з невдачами терапії схемами, що містять інтерферон, використано аналіз за допомогою ROC-кривих. Оцінено середній рівень експресії мікроРНК-196а у хворих на ХВГС (Me). Так, у 1-й групі пацієнтів він становив 0,011 (IQR: 0,002; 0,310) і в групі порівняння – 0,346 (IQR: 0,054; 1,239) при p=0,012 за критерієм U. Проведений ROC-аналіз показав, що досліджувана мікроРНК-196а може диференціювати пацієнтів з ХВГС з 1-м генотипом HCV, залежно від попереднього досвіду противірусної терапії, а саме, у хворих з невдачами терапії схемами, що містять інтерферон, та наївних пацієнтів - AUC=0,688 (95% CI 0,570–0,791; p=0,017), J=0,40, Se=57,14%, Sp=83,02%, ДЕ=70,08 пов'язаний критерій експресії miR-196a становив ≤0,0178 ум. од. та для Log_{10} miR-196a був <-1,75 ум. од., що в подальшому може бути використано для скринінгу і дає додаткові можливості для корекції лікувальної тактики хворим. Високоспецифічний критерій класифікатора експресії miR-196a становив ≤0,0017 ум. од. (Se=28,57%, Sp=98,11%, ДЕ=63,34%), що дає додаткові можливості для корекції лікувальної тактики хворим. Таким чином, рівень експресії мікроРНК-196а може бути додатковим біомаркером у патогенезі ХВГС, що в подальшому може бути застосовано при моніторингу і лікуванні хворих. Низький рівень експресії мікроРНК-196а, а саме ≤0,0017 ум. од. (Log_{10} <-2,78 ум. од.), може бути підґрунтям для призначення хворим більш ефективних схем терапії із застосуванням противірусних препаратів прямої дії та дозволить персоніфікувати лікувальну тактику у хворих на ХВГС.

The incidence of chronic viral hepatitis C (hepatitis C) remains high in the world and in Ukraine. The incidence rate in the Dnipropetrovsk region continues to increase from year to year [2, 7]. Much effort is being made in the world to eliminate viral hepatitis, so a comprehensive study of the pathogenesis of HCV infection, the likely impact on the virus and / or its host factors, and the prognosis of the disease are still relevant. One direction of epigenetics is the expression of RNA that does not encode a protein, namely, miRNAs. MicroRNAs are molecules with a length of about 18-22 nucleotides, they play a crucial role in the regulation of gene expression [4]. Extracellular miRNAs in body fluids are stable under harsh conditions, including boiling, low / high pH, long-term storage and several freeze-thaw cycles [8]. MiRBase is a major public repository and online resource for miRNA sequences and annotations. Today the family of microRNAs has expanded to more than 1,900 annotated precursor miRNAs and 2,654 mature human miRNA sequences (miRBase v22.1; http://www.mirbase.org) [8].

The functions of miRNAs are studied both experimentally, on cell culture, and in patients with a particular pathology. A number of micro-RNAs are a vital component of the innate antiviral immune response. Many microRNAs are involved in both cancers and tumor suppressor genes in various cancers. HCV-infection causes chronic inflammation, and the regulation of inflammation associated with miRNAs promotes the initiation and progression of hepatocellular carcinoma [7].

There are publications examining the level of expression of various miRNAs in patients with hepatitis C or in the culture of liver cells infected with HCV in an experimental model. It has been found that interferon-α/β (IFN-α/β) rapidly induces the expression of certain cellular miRNAs (miR-1, miR-30, miR-128, miR-196, miR-296, miR-351, miR-431 and miR-448, which showed similar complementarity in their sequences with HCV RNA genomes in the HCV infection model) and suppresses miR-122. These results not only offer a new model of host defense mechanisms that exist in mammalian cells, but also add a new component to the antiviral arsenal that uses interferons [6]. It has been studied that miR-122 promotes HCV replication in infected cells, and increased expression of miR-448, miR-196, Let-7b, on the contrary, inhibit viral replication by directly acting on the HCV genome in an experimental model [5, 9]. Increased expression of miR-199a inhibited the replication of HCV-1b or -2a genotypes in cells. Gene expression analysis revealed changes in the regulation of miR-449a in patients with chronic viral hepatitis C [8]. Other authors have studied the profiles of 27 miRNAs in 106 Egyptian patients with the 4th genotype of HCV-infection [12] and in 12 Italian patients (miR-1, miR-30, miR-128, miR-196, miR-296) [6], including, depending on response to antiviral therapy by interferon-containing regimens and revealed various changes in miRNA profiles after interferon induction. Recent publications have cited about 100 microRNAs that are specific for the liver in various conditions. Thus, different expression levels of miR-122, miR-130, miR-183, miR-196, miR-209 and miR-96 are potential indicators of liver damage (via apoptosis, necrosis and necroptosis) during acute /

fulminant or chronic hepatitis, fibrosis / cirrhosis of the liver and hepatocellular carcinoma [11]. However, the function of many miRNAs in chronic viral hepatitis C has not yet been fully understood.

Further direction of science development is the use of epigenetic therapy as a new direction in the treatment of diseases, especially in oncology and infectology [4, 10]. Therefore, the level of expression and function of miR-196a in hepatitis C has not yet been fully established and studied in the Ukrainian cohort of patients.

The aim of the study was to investigate the level of expression of miR-196a in Ukrainian patients with chronic viral hepatitis C with HCV genotype 1, depending on previous experience of interferon-containing antiviral therapy to predict the likelihood of an unsuccessful response to antiviral therapy and further assigning optimal therapy regimens to problem patients.

MATERIALS AND METHODS OF RESEARCH

The study involved 74 patients with chronic viral hepatitis C with 1st HCV genotype with a mean age of 47.5±1.4 years. Of these, 38 were men (51.4%), 36 were women (48.6%). The average duration of the disease since the patient was first diagnosed with chronic viral hepatitis C (Me) was 4.0 years (IQR: 2.0; 8.0) and in some cases – up to 27 years. Patients were monitored at the Hepatology Department of Dnipro City Clinical Hospital N 21 and were examined in accordance with clinical protocols and bioethical standards. General clinical and biochemical blood tests, genotype of hepatitis C virus (HCV), viral load of HCV, level of liver fibrosis, etc were performed in patients. The level of fibrosis was determined using non-invasive methods that correspond to the assessment of the degree of fibrosis by the METAVIR scale. Namely, according to laboratory indicators of FibroTest® (BioPredictive, France) and / or evaluation of the stiffness (elasticity) of the liver tissue by the instrumental method, by various methods of ultrasonic compressional elastography of the liver.

To study the expression level of miR-196a (synonyms: miR-196a, hsa-miRNA-196a), a two-step study was used according to the manufacturer's protocol based on the Department of General and Molecular Pathophysiology of Bogomoletz Institute of Physiology of NAS of Ukraine (Head - Professor A.A. Krishtal, head of the Department – Professor V.E. Dosenko). First, total RNA was isolated from the blood plasma by phenol-chloroform extraction. Then, to evaluate the level of mature miRNAs, reverse transcription was performed using a TaqMan® microarray reverse transcription kit (Applied Biosystems, USA), specific loop primers for mature miRNA-196a, or snRNA U6 (as endogenous control gene). Real-time quantitative PCR was performed using TaqMan® microRNA analysis (Applied Biosystems, USA): hsa-miR-196a and sn6 URNA. The level of miRNA was calculated by the formula ($2^{-\Delta Ct}$), normalized to U6 snRNA and represented in conventional units (C.U.).

Data processing and analysis were performed using Statistica v.6.1® software (StatSoft, USA, serial number AGAR909E415822FA), MedCals v.19.0.7 (free trial; access mode: https://medcalc.org). Quantitative data are presented as a range of values (minimum-maximum), arithmetic mean and standard error (M±m) at normal distribution (Shapiro-Wilk test), and as median (Me) and interquartile range (IQR: Q25; Q75) – in other cases. The Student's (t) and Mann-Whitney (U) criteria were used to compare the averages, for the relative values – Fisher's two-sided eriterion FET for the 2x2 conjugation tables and Pearson's χ^2 in other cases. Diagnostic value of miR-196a expression level to optimize the prognosis of response to antiviral therapy and to administer optimal therapy regimens for problem patients with interferon-containing treatment regimens was determined using ROC curves with the calculation of ROC-operating characteristics: Area under ROC curve – (AUC) with confidence intervals (CI 95%), Youden index (J), sensitivity (Se), specificity (Sp) and diagnostic efficiency (DE) of the model [1]. The critical level of statistical significance (P value) was assumed to be <5% ($p<0.05$).

RESULTS AND DISCUSSION

Following clinical anamnestic analysis, laboratory and instrumental studies, baseline expression of miR-196a was determined in all patients compared to healthy subjects, as reported in our previous publications [3]. The patients were divided into two groups, depending on previous experience of antiviral therapy (AVT) with interferon-containing regimens (IFN-containing regimens). The first group included 21 patients with AVT failure with interferon-containing regimens (AVT failures). Namely, 4 (19.0%) patients did not respond, 3 (14.3%) patients had a partial virological response (14.3%), recurrence of the disease after 6 months or more at the end of therapy – 14 (66.7%) patients. Patients received AVT from 1 to 7 years ago, on average – 2 (2; 3) years ago. The second group (comparison group) consisted of 53 naive patients (without previous experience of therapy). Both groups were statistically comparable by gender ($p=0.610$ by FET criterion) and by age ($p=0.074$ t). General characteristics of the groups, the main demographic and clinical laboratory parameters of patients are presented in Table.

Socio-demographic and clinical-laboratory parameters of patients with chronic viral hepatitis C and the first genotype

Characteristics	Patients with chronic viral hepatitis C (n=74)		
	group 1 (n=21)	group 2 (n=53)	P value between groups
Age, years, M ± m (range)	42.5±2.8 (18 – 62)	49,4±1,5 (23 – 70)	0.074
Duration of disease since first diagnosis of CVHC, Me (IQR) (range)	5.0 (3.0; 9.0) (2-19)	4.0 (1.0; 8.0) (0-27)	0.078
Body mass index (BMI), M ± m (range)	27.7±1.2 (19.3 – 37.9)	27.1±0.7 (19.1 – 39.7)	0.748
Sex, number (%): Men / Women	12 (57.1)/ 9 (42.9)	26 (49.1)/ 27 (50.9)	0.610
ALT, U/ml, Me (IQR)	41.4 (35.2; 71.0)	67.0 (37.1; 111.2)	0.120
AST, U/ml Me (IQR)	37.2 (28.2; 65.2)	53.5 (35.4; 81.0)	0.111
Bilirubin, mcmol/l, Me (IQR)	12.3 (10.6; 18.0)	15.0 (11.5; 20.6)	0.334
Creatinine, mmol/l, Me (IQR)	83.4 (72.0; 91.7)	81.0 (72.0; 93.0)	0.924
RNA HCV, log_{10} copies/ml, M ± m (range) Me (IQR)	6.39±0.15 (4.72-7.48) 6.48 (6.03; 6.66)	6.52±0.09 (5.16 – 7.72) 6.61 (6.09; 7.01)	0.257
Stages of fibrosis by METAVIR, number (%) F1 F2 F3 F4	 8 (38.1) 7 (33.3) 2 (9.5) 4 (19.0)	 17 (32.1) 14 (26.4) 9 (17.0) 13 (24.5)	0.759

As can be seen from Table, the patients of both groups had no statistically significant differences (p>0.05) in terms of the main demographic and laboratory parameters – total blood count, biochemical parameters of the liver complex, body mass index (BMI), etc.

In special studies, after isolation of total RNA in the blood plasma, control gene U6 was isolated, the level of expression does not depend on any pathological conditions of the body. Initially, the level of expression of the U6 gene in the study groups was studied; median level in all patients with chronic viral hepatitis C was (27.31±0.5) conventional units (C.U.), including patients of group 1 (27.38±1.06) C. U., in patients of group 2 (27.28±0.58) C.U., with no significant differences between groups (p=0.919 by criterion U).

A study of the level of expression of miR-196a in groups of patients with hepatitis C showed their considerable variability. The indicator ranged from $4.67*10^{-6}$ to 26.65 C.U. in the 1st group and from 4.67*10-5 to 27.06 in the 2nd group. The median expression level of miR-196a in patients of group 1 was 0.011 (IQR: 0.002; 0.310) C.U. and in the comparison group – 0.346 (IQR: 0.054; 1.239) at p=0.012 according to the U criterion (Fig. 1a). Decimal logarithms of indicators in patients of group 1 averaged 1.94 (IQR: -2.78; -0.51) C.U. and in patients in group 2 – 0.46 (IQR: -1.27; 0.09) (Fig. 1b).

As can be seen from Figure 1, a significant difference (p=0.012) in the level of expression of miR-196a between patients with hepatitis C in the naive group and with failured on AVT to IFN-containing regimens may reflect a potential mechanism of HCV persistence. A significant decrease in the expression level of miR-196a may be an additional biomarker in predicting the effects of treatment with IFN-containing regimens. The data obtained can be compared with studies by other authors, where the level of expression of miR-196a differed in 12 patients with chronic viral hepatitis C – responders and with the failure of AVT to interferon therapy [6], and suggest that in group of naive patients a greater proportion of patients with with a favorable genotype of interleukin 28-β (against unfavorable genotype in non-responders) and other factors and a high likelihood of responding to IFN-containing AVT regimens.

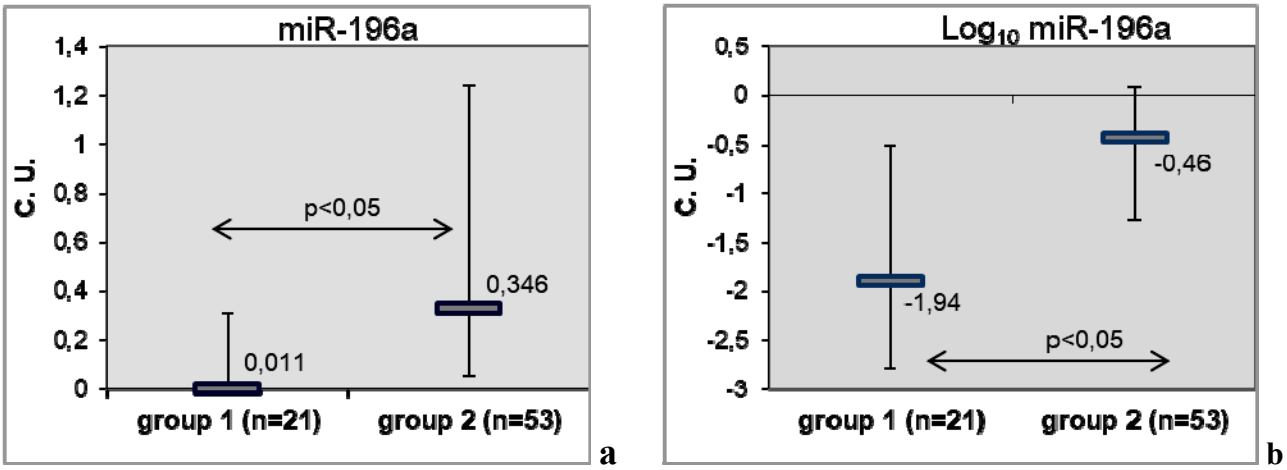

Fig. 1. (1a, 1b) Medium expression of miR-196a in the blood of patients with chronic viral hepatitis C (1a – in C.U.; 1b – in log_{10} C.U.): Me (IQR) indicated, C.U. - conventional units

In order to determine the critical levels of expression of miR-196a in patients with chronic viral hepatitis C, which increase the likelihood of a negative response to AVT with IFN-containing regimens and the subsequent administering of optimal therapy regimens to problem patients, a ROC analysis was performed (Fig. 2).

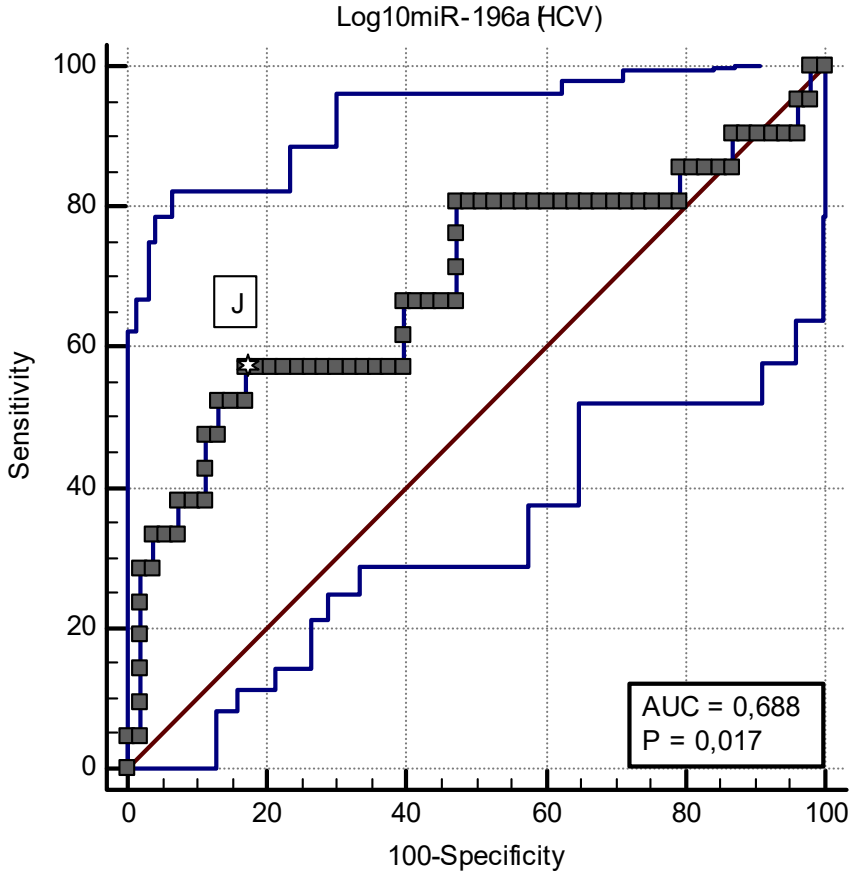

Fig. 2 - ROC curves with CI 95% of estimate of miR-196a expression level in blood of patients with CVH C and Youden index (J), depending on previous experience of antiviral therapy with interferon-containing regimens

According to the results of ROC analysis (Fig. 2), it was found that studied miR-196a can differentiate patients with 1st genotype of hepatitis C, depending on previous experience of antiviral therapy, namely, in patients with failure of IFN-containing regimens and naive patients with an area limited by the ROC curve and the axis of the proportion of false positive classifications, AUC=0.688 (95% CI 0.570-0.791; p=0.017), J=0.40, indicating the average quality of this classifier. Critical cut-off points for patients with probable AVT failure of IFN-containing regimens is the expression level of miR-196a ≤0.0178 C.U., for Log10 miR-196a <-1.75 C.U. The sensitivity and specificity of the model for differentiation of failure in the application of interferon schemes were Se=57.14% and Sp=83.02%, DE=70.08. These results allow to admit that the miR-196a may be a potential diagnostic biomarker in patients with chronic hepatitis C with the 1st HCV genotype.

The optimal highly specific criterion for the miR-196a expression classifier is points ≤0.0017 C.U. and <-2,78 C.U. for Log10 miR-196a with Se = 28.57%, Sp=98.11%, DE=63.34%. These criteria may be the basis for prescribing more effective treatment regimens.

Thus, our identified profile of miR-196a expression level, depending on previous treatment experience in Ukrainian patients with HCV genotype 1, may be useful in patients with chronic viral hepatitis C both in Ukraine and in the world. Along with the main clinical and laboratory indicators, the severity criteria for chronic viral hepatitis C and known prognostic markers for response to antiviral therapy, epigenetic markers, such as the level of expression of miR-196a, will be taken into account, which will allow personification of therapeutic tactics in patients with chronic viral hepatitis C, especially at the choice of the schemas of antiviral therapy depending on the presence of interferon in the scheme.

CONCLUSIONS

1. The performed study showed that the average expression of miR-196a (Me) in Ukrainian patients with the first HCV genotype of chronic viral hepatitis C with unsuccessful previous experience with interferon-containing antiviral regimens was 0.011 (IQR: 0.002; 0.310) C.U. and was significantly (p<0.05) below the indicator in naive patients - 0.35 (IQR: 0.05; 1.24) C.U., which may be an additional biomarker in the pathogenesis of chronic viral hepatitis C to determine the effectiveness of therapy.
2. Critical expression levels of miR-196a ≤0.0178 C.U. and <-1.75 C.U. for Log10 miR-196a were established by ROC analysis and provide the possibility of differentiation of patients with the 1st HCV genotype of chronic viral hepatitis C depending on previous experience of antiviral therapy with test performance Se=57.14%, Sp=83.02%, DE=70.08%, which may be used for screening and provides additional opportunities for correction of therapeutic tactics for patients.
3. Low level of expression of miR-196a, namely ≤0.0017 C.U. (Log10<-2.78 ppm) may be the basis for prescribing more effective treatment regimens using direct-acting antiviral drugs to naive patients and will allow personification of therapeutic tactics in patients with chronic viral hepatitis C.

Prospects for further research. Given the low diagnostic information of the ROC-determined level of expression of miR-196a for predicting the response to IFN-containing AVTs, and to improve the accuracy of the method, it is promising to consider this marker along with the level of expression of other miRNAs and factors for optimizing the prediction of problem patients with chronic viral hepatitis C response for prescribing more effective antiviral therapy regimens, both at primary care level and in highly specialized third-level institutions of medical care.

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Conflict of interests. The authors declare no conflict of interest.

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