THE ROLE OF CIRCULATING miR-19b MIRNA IN PREDICTING THE OUTCOME OF COVID-19

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ABSTRACT

Background. *MicroRNAs are short (20–22 nucleotides) non-coding RNAs that can posttranscriptionally regulate gene expression and are considered a regulator of the innate immunity system. Previously, many papers were published on the prediction of the interaction of the single-stranded (+)RNA virus SARS-CoV-2 with human microRNAs, as well as on the profile of circulating microRNAs in patients with COVID-19 of varying severity. However, no works are analyzing the possible contribution of miRNAs circulating in blood plasma to the severity of COVID-19.*
The aim. *To study the features of the blood plasma microRNA profile of patients with different severity of the new coronavirus infection COVID-19 and to evaluate the possibility of microRNA interaction with the SARS-CoV-2 genome.*
Materials and methods. *The results of NGS sequencing of plasma miRNAs of 3 recovered and 8 deceased patients with a highly severe form of COVID-19 were studied. Differentially presented microRNAs were determined using bioinformatics methods, and their binding sites with the SARS-CoV-2 genome were predicted.*
Results. *This study demonstrates that in patients who have recovered from a highly severe form of COVID-19, the level of hsa-miR-19b-3p in the blood plasma is significantly increased. This microRNA makes up about 1.5 % of all circulating microRNAs and can bind to SARS-CoV-2 regions encoding proteins that suppress intracellular immunity mechanisms (NSP3, NSP9). In addition, this miRNA can stimulate the functional activity and proliferation of cytotoxic T-lymphocytes, one of the critical components of acquired cellular immunity against SARS-CoV-2.*
Conclusion. *The results of the study can be used in the development of antiviral drugs based on RNA interference, as well as in the development of predictive test systems to optimize the tactics of treating patients with COVID-19.*

Key words: *miRNA, COVID-19, SARS-CoV-2, miR-19b, disease severity*

Received: 03.04.2023
Accepted: 18.04.2023
Published: 05.05.2023

For citation: Shkurnikov M.Yu., Kolesnikov S.I. The role of circulating miR-19b miRNA in predicting the outcome of COVID-19. *Acta biomedica scientifica*. 2023; 8(2): 26-32. doi: 10.29413/ABS.2023-8.2.3

РОЛЬ ЦИРКУЛИРУЮЩЕЙ МИКРОРНК miR-19b В ПРОГНОЗЕ ИСХОДА COVID-19

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РЕЗЮМЕ

Обоснование. *МикроРНК – короткие (20–22 нуклеотида) некодирующие РНК, обладающие способностью постранскрипционно регулировать экспрессию генов, рассматриваются в качестве регулятора системы врождённого иммунитета. Ранее был опубликован ряд работ, посвящённых предсказанию взаимодействия одноцепочечного (+)РНК-вируса SARS-CoV-2 с микроРНК человека, а также особенностям профиля циркулирующих микроРНК у пациентов с COVID-19 различной степени тяжести. Однако практически отсутствуют работы, анализирующие возможный вклад фактически циркулирующих в плазме крови микроРНК в тяжесть течения COVID-19.*

Цель. *Изучить особенности профиля микроРНК плазмы крови пациентов с различной тяжестью течения новой коронавирусной инфекции COVID-19 и оценить возможность взаимодействия микроРНК с геномом SARS-CoV-2.*

Материалы и методы. *Изучены результаты NGS-секвенирования микроРНК плазмы 3 выздоровевших и 8 умерших пациентов с крайне тяжёлой формой COVID-19. С помощью биоинформационных методов определены дифференциально представленные микроРНК, предсказаны места их связывания с геномом SARS-CoV-2.*

Результаты. *В данной работе продемонстрировано, что у пациентов, выздоровевших после крайне тяжёлой формы COVID-19, в плазме крови статистически значимо повышен уровень hsa-miR-19b-3p. Данная микроРНК составляет около 1,5 % от всех циркулирующих микроРНК, способна связываться с регионами SARS-CoV-2, кодирующими белки, подавляющие внутриклеточные механизмы иммунитета (NSP3, NSP9). Кроме того, данная микроРНК способна стимулировать функциональную активность и пролиферацию цитотоксических Т-лимфоцитов – одного из ключевых компонентов приобретённого клеточного иммунитета против SARS-CoV-2.*

Заключение. *Результаты исследования могут быть использованы при разработке противовирусных препаратов на основе РНК-интерференции, а также при разработке прогностических тест-систем для оптимизации тактики лечения пациентов с COVID-19.*

Ключевые слова: *miRNA, COVID-19, SARS-CoV-2, miR-19b, тяжесть течения заболевания*

Статья получена: 03.04.2023
Статья принята: 18.04.2023
Статья опубликована: 05.05.2023

Для цитирования: Шкурников М.Ю., Колесников С.И. Роль циркулирующей микроРНК miR-19b в прогнозе исхода COVID-19. *Acta biomedica scientifica*. 2023; 8(2): 26-32. doi: 10.29413/ABS.2023-8.2.3

OBJECTIVES

In 2019, a new disease called COVID-19, caused by the SARS-CoV-2 coronavirus, was identified. This virus was characterised by rapid, difficult to control spread due to its certain characteristics. The body's first line of defence against viruses, including SARS-CoV-2, is innate immunity, which limits viral entry into cells, translation, replication and virion assembly, and allows detection and destruction of infected cells, coordinates and enhances acquired immunity [1]. Innate antiviral immunity has two components: cellular and humoral immunity, which include macrophages, monocytes, dendritic cells, neutrophils, NK cells, cytokines and interferons, and intracellular immunity. Intracellular immunity includes various families of cytoplasmic receptors and enzymes that can recognise and destroy viruses within a cell [2].

Several studies consider microRNA molecules as a component of the innate immunity system [3–5]. MicroRNAs are short (about 22 nucleotides) non-coding RNAs that regulate gene expression post-transcriptionally. The microRNA sequence includes the so-called seed region, which is located from the 2nd to the 7th nucleotide from the 5'-end of the mature molecule, and which is responsible for the specificity of its binding to target RNAs. It has been shown that host cell microRNAs can act as a component of intracellular immunity, regulating the translation and replication of (+)RNA viruses and altering the pathogenesis of viral infections [6, 7]. Two main effects of the interaction between the viral RNA-genome and microRNA are identified: inhibition of virus translation and slowing down its replication, stabilisation of viral RNA and increasing the rate of virus replication. Moreover, the slowdown of virus replication is primarily associated with the interaction of microRNA with the 3'-untranslated region of the virus [8], and the stabilisation of viral RNA is associated with the interaction with the 5'-untranslated region of the virus [9]. Moreover, microRNAs are able to modulate the activity of both innate [10] and acquired immunity cells [11].

A number of papers have been published on predicting the interaction of the single-stranded (+) RNA virus SARS-CoV-2 with human microRNAs [12–14], as well as the profile of circulating microRNAs among patients with different degrees of COVID-19 severity [15]. However, there are practically no works analysing the possible contribution of microRNAs circulating in plasma to SARS-CoV-2 biogenesis and COVID-19 pathogenesis.

THE AIM OF THE STUDY

To study the features of the blood plasma microRNA profile of patients with different severity of the new coronavirus infection COVID-19 and to evaluate the possibility of microRNA interaction with the SARS-CoV-2 genome.

METHODS

Plasma microRNA profile of patients with COVID-19

Primary microRNA sequencing data (GSE195898) isolated from blood plasma of 3 recovered (two men and one woman) and 8 deceased patients (five men and three women, comparison group) with extremely severe COVID-19, comparable in sex and age, treated at the IRCCS Policlinico San Donato Intensive Care Unit (Milan, Italy) were used for analysis [16]. For each patient, the plasma microRNA profile was assessed at the time of hospital admission (T0) and before discharge or death (T1).

The study was carried out in full compliance with the World Medical Association Declaration of Helsinki. The experimental protocol was approved by the local ethical committee at San Raffaele Hospital (Milan, Italy, Minutes No. 75/INT/2020 dated April 20, 2020).

HTG EdgeSeq miRNA Whole Transcriptome targeted sequencing kit (HTG WTA, HTG Molecular, USA) was used for library preparation. Sequencing was performed on an Illumina NextSeq 500 sequencer (Illumina Inc., USA) using the NextSeq High Output v. 2 75 cycles kit (Illumina Inc., USA) [16].

Sequencing data analysis

The 3'-adapter sequence was removed using Cutadapt v. 2.10. Sequencing read quality control was performed using FastQC v. 0.11.9. After removal of the 3'-adapter sequence, sequencing results were processed using IsoMiRmap [17].

Identification of microRNA binding motifs to the SARS-CoV-2 genome

For each microRNA, SARS-CoV-2 RNA regions reverse-complementary to the 2–7 nucleotide region of the 5'-end of the mature microRNA were identified. Following a common classification [18], such binding regions are labelled "6mer". Interaction of the corresponding microRNA with them leads to suppression of translation and degradation of the target RNA. To determine whether 6mer belongs to regions encoding SARS-CoV-2 proteins, the binding positions were pairwise aligned against the reference sequence of the Wuhan-Hu-1 strain using the stringr and spgs packages.

Statistical analysis

Differences in microRNA representation were analysed using DESeq2 [19]. Comparison of data on the number of microRNA binding motifs to the SARS-CoV-2 genome was performed using the Wilcoxon test. Data processing and statistical analyses were performed in the *R* software environment.

RESULTS

Sequencing results showed that 932 types of microRNAs and their 5'-isoforms were present in the patients' plasma at more than 150 rpm at the time of admission (T0). At T1, 990 types of molecules were identified. Moreover, 54 types of molecules occurred only at T0 and 112 occurred only at T1. The following microRNAs are among the most highly represented at T0 and T1: hsa-miR-22-3p|0, hsa-miR-339-3p|0, hsa-miR-451a|0.

Analysis of differences in microRNA profile between recovered and non-recovered patients showed that the ex-

pression of 46 microRNAs was significantly altered. At T0, subsequently recovered patients had higher expression of hsa-miR-19b-3p|0 (4.5-fold, $p = 0.017$), hsa-miR-25-3p|+1 (4.8-fold; $p = 0.047$). The representation of 291 microRNAs differed significantly at T1. The largest differences in plasma representation between the group of recovered patients and the comparison group were observed for the following microRNAs: hsa-miR-451a|0 (13-fold; $p = 7.65E-07$), hsa-miR-22-3p|0 (4.3-fold; $p = 7.67E-05$), hsa-miR-19b-3p|0 (14-fold; $p = 1.23E-06$).

Several studies have demonstrated the correlation of hsa-miR-451a representation and the level of haemolysis in blood samples [20, 21]. The hypothesis of significance of differences in the representation of microRNAs associated with haemolysis between the comparison groups at T0 and T1 was tested (Table 1). At the time of hospital admission, the levels of microRNAs associated with haemolysis and erythropoiesis did not differ between the comparison groups. Meanwhile, all microRNAs associated with haemolysis and erythropoiesis were significantly elevated at T1 in the group of recovered patients.

Comparison of microRNA sets differing between recovered patients and the comparison group showed that only two microRNAs changed codirectionally at T0 and T1 (Table 2).

The possible binding sites of multiple microRNAs differing between the convalescent patient group and the comparison group at T0 and T1 to the SARS-CoV-2 genome were evaluated (Figure 1). A number of microRNAs had no binding sites with the virus genome: hsa-miR-1225-3p|+3, hsa-miR-4498|+1, hsa-miR-6787-5p|+2, hsa-miR-1538|+1, hsa-miR-1307-5p|+1, hsa-miR-7111-5p|+2. The number of microRNA binding sites of hsa-miR-19b-3p|0 and hsa-miR-25-3p|+1 were 12 and 9, respectively. At the same time, the median of binding sites of the other microRNAs was 3. It can be concluded that recovered patients had a significantly

TABLE 1

DIFFERENCES IN THE REPRESENTATION OF MICRORNAS ASSOCIATED WITH HEMOLYSIS BETWEEN THE COMPARISON GROUPS IN T0 AND T1

MicroRNAs	MicroRNA representation in plasma at T0, log2RPM			MicroRNA representation in plasma at T1, log2RPM		
	recovered	deceased	*p value*	recovered	deceased	*p value*
hsa-miR-451a|0	14.4 ± 2.2	13.2 ± 1.7	0.0959	17.4 ± 1.8	13.9 ± 1.6	1.0E-06
hsa-miR-16-5p|0	11.6 ± 1.5	11.6 ± 1.3	0.9370	13.9 ± 1.3	12.1 ± 0.8	2.0E-04
hsa-miR-486-5p|0	11.2 ± 2.8	10.3 ± 1.5	0.1206	13.7 ± 1.8	10.5 ± 1.3	1.0E-05
hsa-miR-93-5p|0	10.7 ± 1.9	10.1 ± 1.2	0.1771	12.8 ± 1.3	10.1 ± 0.9	4.0E-07
hsa-miR-17-5p|0	9.3 ± 1.4	9 ± 1	0.4637	11 ± 1.1	9.4 ± 0.7	4.0E-04
hsa-miR-20a-5p|0	8.9 ± 1	8.9 ± 1	0.7719	11.1 ± 1.1	9.4 ± 0.8	2.0E-04
hsa-miR-107|0	8.8 ± 1.2	8.8 ± 0.7	0.7764	10.2 ± 1.1	9.1 ± 0.4	1.4E-03
hsa-miR-106a-5p|0	8.5 ± 1.2	7.9 ± 0.7	0.1640	10.2 ± 1.3	8.2 ± 0.7	2.0E-05
hsa-miR-20b-5p|0	7.8 ± 0.4	7.6 ± 0.3	0.6410	9.1 ± 0.7	7.7 ± 0.5	2.0E-04

TABLE 2

REPRESENTATION OF CONCOMITANTLY CHANGED MIRNAS IN COMPARISON GROUPS IN T0 AND T1

MicroRNAs	MicroRNA representation in plasma at T0, log2RPM			MicroRNA representation in plasma at T1, log2RPM			
	recovered	deceased	*p value*	recovered	deceased	p *value*	p *value* adjusted for multiple comparisons
hsa-miR-19b-3p|0	11.7 ± 2.9	10.6 ± 1.5	0.017	14.3 ± 1.8	10.6 ± 1.4	1.23E-06	0.003
hsa-miR-25-3p|+1	3.4 ± 1.2	1.9 ± 0.8	0.047	4.7 ± 1.2	2 ± 0.7	2.25E-05	0.046

increased level of microRNAs with a significant number of binding sites to the SARS-CoV-2 genome ($p = 0.048$).

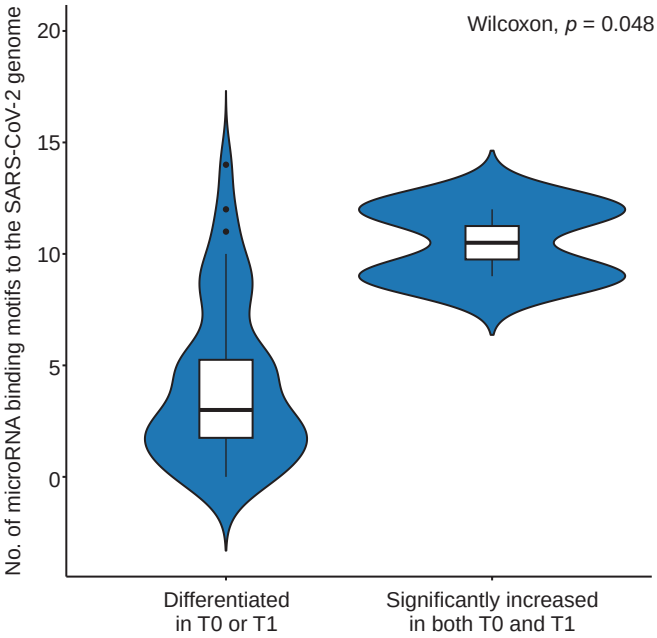

FIG. 1.
The number of possible binding sites of miRNAs in the SARS-CoV-2 genome

The location of possible binding sites of microRNAs to the virus genome was also analysed (Figure 2). The highest number of binding sites was located in the ORF1ab region encoding non-structural proteins. hsa-miR-19b-3p|0 had the highest number of binding sites in the short region (338 nucleotides) encoding the NSP9 protein. Both microRNAs analysed had two possible binding sites each with the extended region (5834 nucleotides) encoding the NSP3 protein. None of the microRNAs had binding sites in the 5`- and 3`-untranslated regions of the SARS-CoV-2 genome.

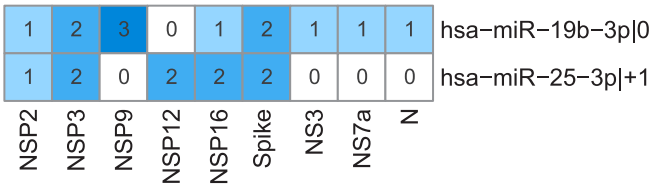

FIG. 2.
Location of possible miRNA binding sites in the SARS-CoV-2 genome

DISCUSSION

Study main result summary

The representation of hsa-miR-19b-3p|0 and hsa-miR-25-3p|+1 microRNAs differed significantly in the plasma of hospitalised patients with extremely severe COVID-19, both at the time of hospital admission and at the time of discharge or death. Both microRNAs have a high number of possible binding sites to the SARS-CoV-2 genome (12 and 9, respectively) compared to other plasma variable microRNAs. The majority of the binding sites are in the parts of the virus genome encoding NSP3 and NSP9.

Study main result discussion

MicroRNAs are short non-coding single-stranded RNA molecules of about 22 nucleotides in length that function as post-transcriptional regulators of gene expression [22]. MicroRNA molecules are involved in many processes including development, proliferation and apoptosis. In addition, microRNAs are associated with many pathological processes [23]. Determination of microRNA expression profiles can act as a method for classifying, diagnosing and predicting disease course [24]. MicroRNAs are detected in various biological fluids and have remarkable stability, emphasising their possible role as promising minimally invasive diagnostic and prognostic markers [25]. Moreover, several studies consider microRNA molecules as a component of the innate immunity system [3–5].

In this study, we studied the profile of circulating microRNAs in the plasma of extremely severe COVID-19 patients. The representation of hsa-miR-19b-3p|0 and hsa-miR-25-3p|+1 differed significantly in the plasma of patients with different COVID-19 outcomes, both at the time of hospital admission and at the time of discharge or death. MicroRNAs can act as a component of intracellular immunity by regulating translation and replication of (+) RNA viruses and altering the pathogenesis of viral infections [6, 7]. Considering that the lung tissue area is 75 to 100 m^2 and that it is abundantly blood supplied [26], it is conceivable that circulating microRNAs could penetrate infected alveocytes and interact with SARS-CoV-2 [27]. hsa-miR-19b-3p|0 significantly elevated in the group of recovered patients is among the most highly represented in plasma. It accounts for more than 1.5 % of all circulating microRNA molecules. This work shows that this microRNA has a significant number of binding sites to the SARS-CoV-2 genome. They are detected in its most stable part which is ORF1ab.

The largest number of hsa-miR-19b-3p|0 binding sites is in the 338-nucleotide long region encoding the NSP9 protein. The NSP9 protein is able to bind to 7SL RNA, component of signal recognition particles, thereby disrupting protein transport into the endoplasmic reticulum and onto the cell membrane [28]. The major histocompatibility complex (MHC) class 1 molecules are produced in the endoplasmic reticulum. Disruption of their synthesis may contribute to impaired antiviral activity of cytotoxic T lymphocytes. In addition, hsa-miR-19b-3p|0 is able to bind to the region encoding the NSP3 protein. NSP3 and NSP4 are responsible for the formation of double-membrane vesicles in the infected cell that protect the virus from the mechanisms of intracellular innate immunity [29]. Thus, hsa-miR-19b-3p|0 highly represented in the plasma of recovered COVID-19 patients is able to bind to virus regions encoding proteins responsible for suppressing intracellular immunity mechanisms.

Moreover, hsa-miR-19b-3p|0 is able to potentiate the activity of cytotoxic T lymphocytes. Levels of hsa-miR-19b-3p|0 were found to be significantly elevated in peripheral blood mononuclear cells of patients in long-term HIV remission. Overexpression of hsa-miR-19b-3p|0 promotes $CD8^+$ T-cell proliferation as well as interferon-γ and granzyme B expression by inhibiting $CD8^+$ T-cell apoptosis induced by anti-CD3/CD28 stimulation. The target of miR-19b was found to be the *PTEN* gene [30].

CONCLUSION

Previously, many papers were published on the prediction of the interaction of the single-stranded (+) RNA virus SARS-CoV-2 with human microRNAs, as well as on the profile of circulating microRNAs in patients with COVID-19 of varying severity. However, there are practically no works analysing the possible contribution of microRNAs circulating in plasma to SARS-CoV-2 biogenesis and COVID-19 pathogenesis. This study demonstrates that in patients who have recovered from a highly severe form of COVID-19, the level of hsa-miR-19b-3p in the blood plasma is statistically significantly increased. This microRNA is present in plasma in significant amounts and is able to bind to SARS-CoV-2 regions encoding proteins that suppress intracellular immune mechanisms. Morevover, it is able to stimulate the functional activity and proliferation of cytotoxic T lymphocytes, a key component of adaptive cellular SARS-CoV-2 immunity. The results of the study can be used in the development of antiviral drugs based on RNA interference, as well as in the development of predictive test systems to optimize the tactics of treating patients with COVID-19.

Financing

The study was supported by a grant from the Ministry of Science and Higher Education of the Russian Federation (Agreement No. 075-15-2021-1049).

Conflict of interest

The authors declare the absence of a conflict of interest.

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