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Original Article

The Expression Level of miR-199a and miR-93 after Hematopoietic Stem Cell Transplantation and Its Relation with Acute Graft-versus-Host Disease, Cytomegalovirus, and COVID-19 Infection

Mahdiyar Iravani Saadi[1], Mani Ramzi[1,2], Ramin Yaghobi[3], Soodabeh Zare[1], Hossain Ali Rostamipour[2,4], Fakhroddin Hosseini[1,2]*

[1]*Hematology Research Center, Shiraz University of Medical Sciences, Shiraz, Iran*
[2]*Department of Hematology, Medical Oncology and Stem Cell Transplantation, Shiraz University of Medical Sciences, Shiraz, Iran*
[3]*Transplant Research Center, Shiraz University of Medical Sciences, Shiraz, Iran*
[4]*Department of Internal Medicine, Jahrom University of Medical Sciences, Jahrom, Iran*

ABSTRACT

Background: The possible role of different microRNAs (miRNAs) as indicators of acute graft-versus-host disease (GVHD) following allogeneic hematopoietic stem cell transplantation (HSCT) has recently come to light. Human cytomegalovirus (CMV) is another common problem that can arise after HSCT. Given human CMV's capacity to remain dormant in human cells indefinitely, the virus has likely refined sophisticated survival mechanisms. miRNAs facilitate one such approach. Coronavirus disease 2019 (COVID-19) is also associated with dysregulation of miRNA expression.

Objective: This study aimed to the expression level of miR-199a and miR-93 after HSCT and its relation with cytomegalovirus and COVID-19 Infection on transplant outcomes.

Methods: One hundred and twenty consecutive patients who received an allogeneic stem cell transplant were enrolled from Namazi Hospital's Transplant Center in Shiraz Iran (2017-2019). In this project, patients are bone marrow transplant recipients. We assessed the expression levels of miR-199a and miR-93 in the peripheral blood of HSCT patients by SYBR Green Real-Time PCR. Cytomegalovirus load was determined via CMV antigenemia assay and COVID-19 load was also determined via Real-time PCR using the COVID-19 kit.

Results: The results showed that miR-93 expression was considerably higher in patients following HSCT (-5.4 1.5 vs. -0.26 0.86; P=0.008). Furthermore, the miR-199a expression level was significantly increased in HSCT CMV+ patients (P=0.005). This study also indicated that the expression of miR-199a was remarkably higher in HSCT patients with COVID-19 (P=0.010).

Conclusion: miR-93 could be a possible prognostic biomarker in patients who had HSCT due to its higher expression in HSCT patients who got aGVHD. Moreover, the level of miR-199a expression in HSCT patients might be related to the pathophysiology of CMV and COVID-19 infection.

KEYWORDS: Hematopoietic stem cell transplantation; miR-199a; miR-93; Cytomegalovirus; COVID-19

INTRODUCTION

MicroRNAs (miRNAs) are involved in the regulation of gene expression in routine biological functions such as cell differentiation, cell death, angiogenesis, and immune cell functions [1]. miRNAs, which are dysregulated in the majority of human diseases, have become a brand-new class of biomarkers with limitless potential [2].

As a curative therapy option, hematopoietic stem cell transplantation (HSCT) that causes the host's HSCs to be replaced by HSCs from a healthy donor or by the patient's own HSCs that have had their genes changed [3]. Acute graft-versus-host disease (aGVHD), which impacts allo-HSCT recipients, is the most common reason for post-transplant mortality

*Correspondence: Fakhroddin Hosseini MD
Hematology Research Center, Shiraz University of Medical Sciences, Shiraz, Iran
ORCID: 0000-0002-3865-0020
E-mail: hosseinif12345@gmail.com

and morbidity [4]. The possible role of different miRNAs as indicators of GVHD following allogeneic HSCT has recently come to light. A four-miRNA plasma profile (miR-423, miR-199, miR-93, and miR-377) has been shown in earlier research to be able to predict the likelihood of aGVHD. Additionally, the severity and survival of aGVHD were linked to the miRNA signature [5]. miRNA levels in body fluids fluctuate during several malignancies and infectious diseases, making it possible to use them as biomarkers for distinguishing between an infected and healthy person [6].

Human cytomegalovirus (CMV) is a common problem that can arise after HSCT. Human CMV-miRNAs are also strong candidates for use as biomarkers in human diseases because of their role in gene regulation of cellular transcripts through gene silencing and the effects of human CMV infection on people with weak immune systems. This is why it's more critical than ever to learn about the human CMV-miRNA expression profile and possible clinical uses [7].

Coronavirus disease 2019 (COVID-19) involvement of the respiratory system is associated with dysregulation of miRNA expression [8]. The miRNA profiles found in this work have the potential to be employed as a new method for early predicting the vital status of COVID-19 patients during illness and prognosis. Very few papers have been written about COVID-19 in combination with miR-199a-5p. Several microRNAs, including miR-199a-5p, were discovered in a study comparing bronchial aspirates from critically sick COVID-19 patients with non-COVID-19 patients [9]. These microRNAs could serve as markers to discriminate between the two samples. miR-199a-5p might be involved in COVID-19 infection [10]. It is believed that this protein has antiviral properties and blocks the virus' secondary envelope. miR-93-3p is a very reliable biomarker. No studies have been conducted in detail on a direct connection between this characteristic and COVID-19. Toll-like receptor 4 (TLR4) is the target of miR-93-3p, which suppresses its expression via a posttranscriptional regulatory mechanism [11]. Due to this, the NF-κB pathway produces more pro-inflammatory factors [12]. In macrophages infected with HIV, miR-93-3p expression was shown to be increased, indicating a potential role of this miRNA in immune responses to viruses [13]. The cardiovascular system is significantly impacted by miR-93-3p, which also causes cytokine storms and symptoms that might lead to myocarditis [14]. In the tissue damage brought on by COVID-19, miR-93-3p might be a crucial factor [10].

We attempted to determine miR-199 and miR-93 prognostic and diagnostic potential by analyzing their expression in the serum of patients who had HSCT at successive time periods from pre-transplant to day 21 post-HSCT. To learn more about their potential significance in the initiation and severity of aGVHD, we also looked at the expression of these miRNAs in the serum of patients. Furthermore, the serum of patients with CMV and COVID-19 infections who underwent HSCT was examined to determine the amount of expression of these miRNAs. The correlation between the expression levels of miR-199a and miR-93 and patient characteristics, blood types, and chemotherapy regimen was then examined.

MATERIALS AND METHODS

Studied Patients

The study included 120 consecutive allogeneic stem cell transplant recipients from Namazi Hospital's Transplant Center in Shiraz Iran (2017-2019). Their ages ranged from five to fifty. For 12 weeks after stem cell transplantation, graft outcome and aGVHD episodes were monitored. Patients were separated into two groups according to whether or not they had aGVHD. The International Bone Marrow Transplant Registry and the traditional Glucksberg-Seattle criteria (GSC) are used to grade acute graft versus host disease (aGVHD) (IBMTR). Specialists have identified the signs and symptoms of blood and marrow transplant failure.

Table 1: The prevalence of the underlying diseases of patients with allogeneic haematopoietic stem cell transplant (HSCT).

Underlying Disease	Number (%)
H. Lymphoma	15 (12.5)
Acute Myeloid Leukemia (AML)	40 (33.3)
Multiple Myeloma	15 (12.5)
N. H. Lymphoma	11 (9.1)
Acute Lymphoblastic Leukemia (ALL)	31 (25.8)
Thalassemia	8 (6.6)

Table 1 illustrates 120 allogeneic HSCT patients' underlying diseases. All but two aplastic anemia patients received peripheral blood hematopoietic stem cells. The standard immunosuppressive protocol for leukemia patients included 16 mg/kg busulfan or busulfan IV (80% of oral dose) and 120–200 mg/kg cyclophosphamide, while severe aplastic anemia and Fanconi's anemia patients were given 60–120 mg/kg and 90 mg/kg ATG, respectively. Cyclosporine and methotrexate prevent GVHD. All patients received preventative antibiotics, antifungals, and antivirals. All blood products were gamma-irradiated to prevent post-transfusion aGVHD. HLA typing was routinely performed in our clinic.

Sample Collection and RNA Extraction

We took five milliliters of peripheral blood from each patient and a healthy person before therapy. EDTA tubes were utilized. In all groups, ficoll-opaque density gradient centrifugation separated mononuclear cells from peripheral blood. Invitrogen's TRIZOL reagent was used to extract total RNA.

Analyze the Expression of miR-93 and miR-199a by SYBR Green Real-time PCR

The SuPrime Script RT Premix (2X) cDNA Synthesis Kit was used to transcribe complementary DNA (cDNA) following the manufacturer's instructions (GeNet BIO Inc; Daejeon, South Korea). The SYBR Green Real-Time PCR technique was used to quantify the level of miR-93 and miR-199a mRNA expression employing SYBR Premix Ex Taq TM II (Tli RNaseH Plus) (Takara, Japan) based on the manufacturer's instructions, as described earlier [15]. mRNA measurement was repeated three times. Internal control was performed using GAPDH. Primer-BLAST was used to design primers for each of miRNA in an iQ5 thermocycler (BioRad Laboratories, USA) (Table 2).

CMV Antigenemia Assay

EDTA whole blood was used for CMV antigenemia testing (IQ Products, Groningen, The Netherlands). Two fluorescein isothiocyanate-labeled monoclonal antibodies (C10/C11) directed against CMV lower matrix protein pp65 was used to stain cytocentrifuge preparations (Cytospin 3, Shandon Scientific, Cheshire, England) of a certain quantity of peripheral blood leukocytes (200,000 cells) by indirect immunofluorescence. The slides were examined under a fluorescence microscope to find leukocytes with uniform brilliant green polylobate nuclear staining. One or more CMV pp65 antigen-infected cells per 50,000 WBCs are considered positive results, which had been established in our previous study [16].

Molecular Detection of COVID-19 Infection

To collect COVID-19 RNA, patients' throats or deep nasal cavities were swabbed. Qiagen's QIAampTM viral RNA small kit was used to extract total RNA from swabs. Swabs were inserted into test tubes with 2 ml of viral transfer media. A real-time PCR COVID-19 kit was used to measure each sample's COVID-19 load (BXM, Iran), which had been adjusted in our previous study [17].

Table 2: Primer sequences for qRT-PCR and used condition for the *MIR-93*, *MIR-199a* and *GAPDH* genes.

Primers	Sequences (5′-3′)	Thermocycling condition
GAPDH	Forward: 5′-GGACTCATGACCACAGTCCA-3′ Reverse: 5′-CCAGTAGAGGCAGGGATGAT-3′	95°C/2 min, 40 cycles of 95°C/30 sec, 57.5°C/20 sec and 70°C/30 sec
MIR-93	Forward: 5′-CGTCCAAAGTGCTGTTCGTGC-3′ Reverse: 5′-GTGCAGGGTCCGAGGT-3′	94°C/2 min, 40 cycles of 94°C/30 sec, 58°C/20 sec and 70°C/30 sec
MIR-199a	Forward: 5′-GATTCCCCAGTGTTCAGACTAC-3′ Reverse: 5′-GTGCAGGGTCCGAGGT-3′	95°C/2 min, 40 cycles of 95°C/30 sec, 60°C/20 sec and 70°C/30 sec

Ethical Considerations

All stages were approved by the Ethics Committee of Shiraz University of Medical Sciences (IR.SUMS.MED.REC.1400.628). In this study, human participation is in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its subsequent amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study.

Statistical Analysis

Data were analyzed using SPSS 18.0. we use paired sample t-test for before-after comparisons, also, an independent t-test was applied to compare miR-93 and miR-199a mean expression levels across patients, controls, and patients depending on treatment response and FAB subtypes. Sample size was calculated based on the Charan and Biswas law and the studies done. The Pearson correlation test was used to compare laboratory results.

RESULTS

Male (M) patients made up 61.3% of allogeneic transplant recipients and female (F) patients were 38.7% ranging in age between 5 and 50 years. The average ages of HSCT patients with aGVHD and those without aGVHD were 33.4±6.1 and 29.3±5.1, respectively. In the aGVHD group, the M/F ratio was 2.1 (38.2), while it was 1.7 (41/23) in the non-aGVHD group. In both allogeneic HSCT patients with and without aGVHD, the most prevalent blood type was O+ (52; 5% and 61; 32.1%, respectively). In 120 allogeneic HSCT cases, the prevalence of the underlying illnesses was shown in Table 1. 46.6% (56/120) aGVHD was found in HSCT recipients. There were 18 of 56 (32.1%) and 38 of 56 (67.8%) HSCT patients with low and severe grades of aGVHD. With an average age of 34.2±8.1 and 25.4±7.2 , respectively).

miR-93 and miR-199a Expression Levels in Patients with HSCT

miR-93 and miR-199a expression levels were evaluated in patients before and after hematopoietic stem-cell transplantation (Fig. 1). The results showed that miR-93 expression was considerably higher in patients following HSCT (-5.4±1.5 vs. -0.3±0.9, P=0.008). However, the expression level of miR-199a was not significantly higher after HSCT (-2.8±1.5 vs. -1.4±0.9, P=0.460).

miR-93 and miR-199a Expression Levels in HSCT Patients with aGVHD

miR-93 and miR-199a mean expression levels were evaluated between HSCT patients with and without aGVHD (Fig. 2). Our findings showed that patients with aGVHD had considerably higher levels of miR-93 expression. Nevertheless, there was no statistically significant difference in miR-199a levels between patients with and without aGVHD (P=0.420). HSCT recipients who experienced low-grade (grade I–II) aGVHD exhibited overexpression of miR-93 and miR-199a in comparison

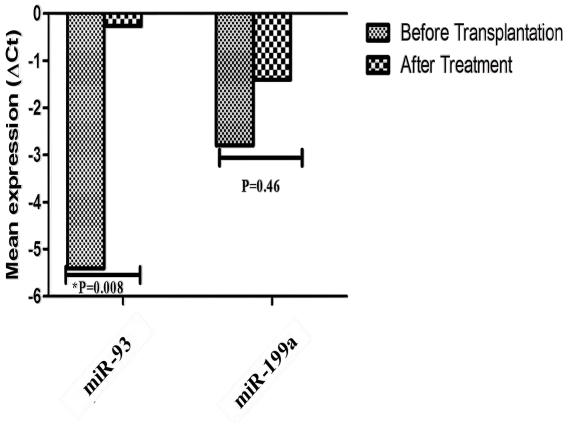

Figure 1: miR-93 and miR-199a expression levels were compared in patients before and after hematopoietic stem cell transplantation. After HSCT, patients' expression levels of miR-93 were significantly higher (-5.4±1.5 vs. -0.3± 0.9, *P=0.008). However, after HSCT, miR-199a expression was not statistically increased (-2.8±1.5 vs. -1.4±0.9, P=0.460).

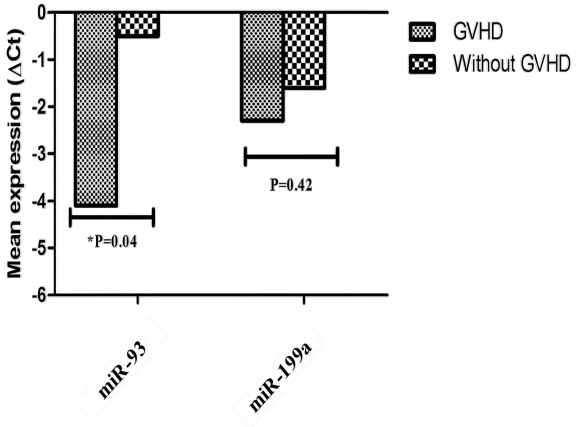

Figure 2: The mean expression levels of miR-93 and miR-199a in HSCT patients with and without aGVHD are compared. miR-93 expression was significantly higher in patients with aGVHD. Despite this, no statistically significant difference in miR-199a levels was seen between patients with and without aGVHD (P>0.05).

to those who experienced high-grade (grade III–IV) aGVHD, however, the difference was not statistically significant (Fig. 3).

Association of miR-93 and miR-199a Expression with CMV Infection in Patients with HSCT

There were 9 CMV patients out of 120 (7.5%) who received HSCT. The mean expression of miR-93 and miR-199a in patients with and without CMV infection was assessed (Fig. 4). According to our findings, only miR-199a expression levels were substantially higher in CMV+ patients compared to CMV- patients (P=0.005). Also, our analysis indicated that the expression of miR93 in these patients was higher in CMV+ patients in comparison to CMV- patients, albeit it was not statistically significant.

Association of miR-93 and miR-199a Expression with COVID-19 Infection in Patients with HSCT

Infection with COVID-19 was found in 8 of 120 patients (6.6%). COVID-19+ patients, compared to COVID-19- patients, had higher miR-93 and miR-199a expression levels, but only the miR199a expression level was significantly higher (P=0.010) (Fig. 5)

Association of WT1, C-kit, and CEBPA with miR-93 and miR-199a in Patients with HSCT

A heatmap was used to show how *WT1*, *C-kit*, and *CEBPA* expression levels in HSCT patients were related to miR-93 and miR-199a (Fig. 6). miR-93 and miR-199a were both related to *C-Kit* expression. Additionally, miR-93 was also associated with *WT1* expression.

DISCUSSION

Currently, in a wide range of illnesses, miRNAs that circulate in serum or plasma serve as useful biomarkers. The expression levels of miR-93 and miR-199a in serum samples from HSCT patients were examined at successive time points from pre-transplant to day 21 post-HSCT to determine their association with disease incidence and severity. Furthermore, we assessed the expression of these miRNAs in serum samples from CMV and COVID-19 patients who had HSCT. The purpose of this effort is to corroborate the findings of previous research that shows the potential of circulating miRNAs as biomarkers. Also, during our previous study, we found that only miR-155 expression level was significantly increased in HBV+ patients compared with HBV- patients [18].

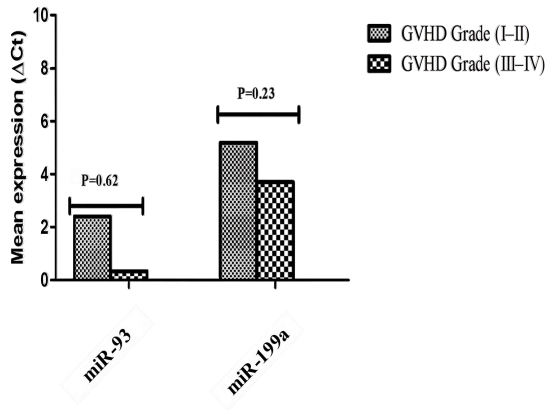

Figure 3: miR-93 and miR-199a expression levels were evaluated in HSCT recipients with varying degrees of GVHD. Patients with low-grade (grade I-II) aGVHD had higher levels of miR-93 and miR-199a expression than those with high-grade (grade III-IV) aGVHD, although the difference was not statistically significant.

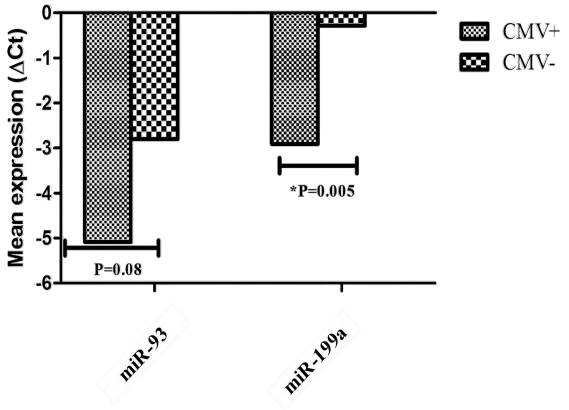

Figure 4: The mean expression of miR-93 and miR-199a in HSCT patients with and without CMV infection was assessed. miR-199a expression levels were substantially higher in CMV+ patients compared to CMV- patients (*P=0.005) and the expression of miR-93 in these patients was higher in CMV+ patients in comparison to CMV- patients, even though it was not statistically significant.

Unfortunately, aGVHD and infectious problems continue to be common HSCT side effects. Clinical characteristics validated by biopsy are the gold standard for diagnosis; hence, biomarkers are required for onset prediction and accurate diagnosis. Proteins are promising plasma indicators [19]. The use of protein-based methodologies, however, can be challenging for therapeutic application as preclinical validation is essential to improving risk stratification or determining individualized treatment. In contrast, miRNAs are robust, express themselves specifically in various tissues and biological stages, and are low in complexity. This makes it possible for them to be identified using standard laboratory techniques [20, 21].

In this study, we found that miR-93 expression was noticeably higher in HSCT patients after transplantation, which is compatible with miR-93's higher expression in aGVHD. This result is consistent with previous research suggesting miR-93 may predict the likelihood of aGVHD and was increased at a median of 16 days before disease onset [5, 22]. In a study by Crossland *et al.* it was determined how miR-423, miR-199, miR-93, and miR-377 were expressed in circulating serum before aGVHD, at the time of the disease's inception, and at subsequent HSCT time points, and how well they predicted the incidence and severity of the condition. They verified the findings of Xiao *et al.* showing that patients who later developed aGVHD had considerably greater expression of miR-423, miR-199, miR-93, and miR-377. Furthermore, in a separate diagnostic cohort made up of serum samples taken at the onset of aGVHD, miR-423, miR-199, and miR-93 had higher levels of expression. However, because miRNAs have tiny seed sequences that could target hundreds of mRNA transcripts, determining their biological relevance may be difficult. So their precise roles in an aGVHD environment have not yet been clarified. miR-93 has been implicated in inflammation, tissue damage, cellular proliferation, and tissue repair [5]. Liu *et al.* showed that miR-93 causes a mesenchymal-epithelial transition in breast cancer cell lines and is associated with the downregulation of numerous stem cell regulatory genes, including *JAK1*, *STAT3*, and *EZH1* [23]. Inhibition of *JAK1* improves mouse survival after aGVHD, lowers histopathological grading, and lowers blood levels of pro-inflammatory cytokines [24]. aGVHD pathogenesis may also be affected by associations with *STAT3*, as this transcription factor increases Treg instability and is elevated before the onset of aGVHD [25]. A conditional reduction of

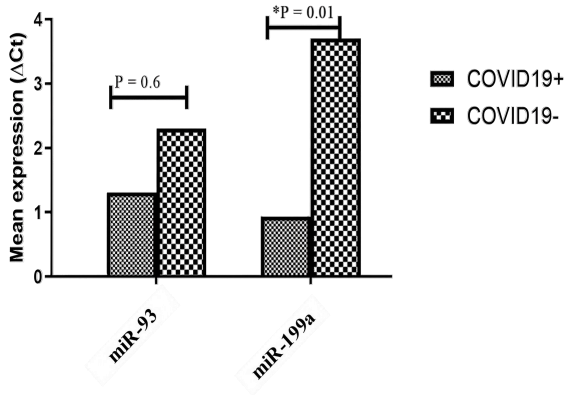

Figure 5: Association of miR-93 and miR-199a expression with COVID-19 infection in HSCT patients. COVID-19+ patients, compared to COVID-19- patients, had higher miR-199a and miR-93 expression levels, but only the miR-199a expression level was significantly higher (*P=0.01).

EZH2 in donor T-cells prevents GVHD in mice, specifically by reducing the ability of T-cells to differentiate into IFN-gamma-producing effector cells, which is another way that *EZH1* is hypothesized to play a role in the development of GVHD [26]. Vascular epithelial growth factor-A (VEGF), a cytokine linked to the prognosis of hematological malignancy, immunological regulation, and tissue healing, has also been shown to be a target of miR-93 in kidney studies [27]. In the case of GVHD, VEGFA levels are linked to incidence, relapse, and non-relapse mortality after HSCT [28].

In this study, miR-93 and miR-199a could not be employed as biomarkers for aGVHD severity in patients undergoing HSCT. In contrast to our findings, some studies revealed that a high level of miR-93 and miR-199 expression was linked to the severity of aGVHD [5, 22].

The amount of particular host miRNAs in a cell has an impact on a variety of aspects of virus-host interactions, and it can also control the potential for viral growth [29]. Only miR-199a expression was substantially higher in CMV+ patients compared to CMV- patients, according to our research. It can raise the risk of other pathogenic infections, morbidity, and death. Our results suggest that miR-199a expression level could be linked to the pathogenesis of CMV infection and could be used as a

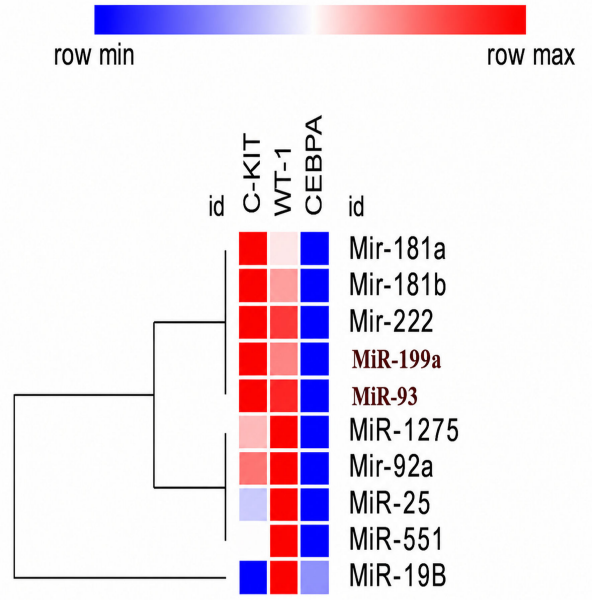

Figure 6: This heatmap was shown how *WT1*, *C-kit*, and *CEBPA* expression levels in HSCT patients were related to miR-93 and miR-199a. They were both connected to *C-Kit* expression. Moreover, miR-93 was associated with *WT1* expression.

biomarker. In line with our findings, CMV infection in endothelial cells increases miR-199a expression and promotes cell migration and tube formation by inhibiting *SIRT1/eNOS* [30]. However, another study found that the miR-199a/miR-214 cluster is downregulated in murine and human CMV infection and has antiviral effects in both mouse and human cells, which is in conflict with our findings [29].

To better understand the SARS-CoV-2 infection pattern and to identify potential treatment options, a variety of approaches have been used. Hsa-miR-199a-3p is one of the six antiviral miRNAs (hsa-miR-1-3p, hsa-miR-17-5p, hsa-miR-429, hsa-miR-15a-5p, and hsa-miR-20a-5p) that have been linked to respiratory illnesses such as influenza A, adenovirus 2, and respiratory syncytial virus (RSV). They are interestingly shown to be downregulated post-infection in a number of lung-damaging viral lung diseases, while in normal cases, these miRNAs are upregulated, suggesting that they could be exploited to design anti-viral medications [31]. ICU stays are necessary for severe SARS-CoV-2 patients, in

which the mortality rate ranges from 25 to 50% [32]. The level of circulating miRNAs expression was compared between ICU and ward patients. Five miRNAs (miR-27a-3p, miR-27b-3p, miR-148a-3p, miR-199a-5p, and miR-491-5p) were found to be upregulated that miR-199a was one of them [32]. Likewise, our findings indicated that miR-199a expression was noticeably higher in HSCT patients with COVID-19 infection compared to those without COVID-19 infection. However, a panel of candidate miRNAs was established in a study by Srivastava *et al.* and hsa-miR-199a-3p was one of eleven miRNAs that were specifically down-regulated in the group of moderate COVID-19 patients [33] that This could be due to viral load or demographic differences in terms of patient type and age. So, the high level of anti-viral miR-199a could predict the incidence and severity of infectious diseases such as COVID-19 infection or CMV.

In conclusion, the miRNA profiles may provide a novel method for predicting the occurrence or severity of aGVHD, CMV, and COVID-19 in HSCT recipients. In HSCT patients, we discovered that miR-199a may be utilized as a biomarker for CMV and COVID-19 and miR-93 was related to the development of aGVHD. However, neither miR-93 nor miR-199a expression levels were able to be employed as biomarkers for the severity of aGVHD. For use of these miRNAs as therapeutic targets validation with a larger cohort may be beneficial.

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CONFLICTS OF INTEREST: None declared.

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