IJMS
Vol 48, No 1, January 2023

Original Article

Expression of MicroRNA-155 in Patients with Non-Hodgkin Lymphoma, Coronavirus Disease 2019, or Both: A Cross-Sectional Study

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Received: 26 June 2021
Revised: 23 November 2021
Accepted: 10 December 2021

Abstract

Background: Non-Hodgkin lymphoma (NHL) is the eleventh leading cause of cancer-related death in the world. Diffuse large B-cell lymphoma (DLBCL) is the most common type of NHL. Up to winter 2021-2022, the death toll caused by the coronavirus disease 2019 (COVID-19) has exceeded 5.6 million worldwide. Possible molecular mechanisms involved in the systemic inflammation, and cytokine storm in COVID-19 patients are still not fully understood. MicroRNA-155 (miR-155) plays a role in the post-transcriptional gene regulation of hematopoiesis, oncogenesis, and inflammation. The present study aimed to evaluate the expression of miR-155 in patients with DLBCL and/or COVID-19.

Methods: A cross-sectional study was conducted from July to December 2020 in Tehran (Iran) to evaluate the expression of miR-155 in adult patients diagnosed with DLBCL and/or COVID-19. The real-time polymerase chain reaction technique was used to evaluate the expression of miR-155 in the sera of 92 adults who were either healthy or suffering from DLBCL and/or COVID-19. Relative quantification of gene expression was calculated in terms of cycle threshold (Ct) value. Data were analyzed using SPSS software, and $P<0.05$ was considered statistically significant.

Results: The expression of miR-155 was not associated with the sex or age of the participants. In comparison with healthy individuals (-ΔCt: -1.92±0.25), the expression of miR-155 increased in patients with COVID-19 (1.95±0.14), DLBCL (2.25±0.16), or both (4.33±0.65).

Conclusion: The expression of miR-155 increased in patients with DLBCL and/or COVID-19.

Please cite this article as: Mahdavi Anari SR, Kheirkhah B, Amini K, Roozafzai F. Expression of MicroRNA-155 in Patients with Non-Hodgkin Lymphoma, Coronavirus Disease 2019, or Both: A Cross-Sectional Study. Iran J Med Sci. 2023;48(1):26-34. doi: 10.30476/IJMS.2022.91669.2282.

Keywords ● COVID-19 ● Inflammation ● MicroRNAs ● Lymphoma ● Non-Hodgkin ● Polymerase chain reaction

What's Known

- MicroRNA-155 plays a role in the post-transcriptional gene regulation of hematopoiesis, oncogenesis, and inflammation.
- Possible molecular mechanisms involved in the systemic inflammation and cytokine storm in patients with coronavirus disease 2019 (COVID-19) are still not fully understood.

What's New

- The expression of microRNA-155 was increased in the sera of patients with diffuse large B-cell lymphoma and/or COVID-2019.

Introduction

Non-Hodgkin lymphoma (NHL) is the seventh most commonly diagnosed cancer and the eleventh leading cause of cancer-related death in the world.[1] In Iran, it accounts for 3%-7% of all diagnosed cancers and is the fifth and eighth most common malignancy in Iranian men and women, respectively.[2] The burden of NHL is set to spiral, particularly diffuse large B-cell lymphoma

(DLBCL).[1, 2] DLBCL is the most common type of NHL in adults, accounting for 40% of all lymphoid cancers worldwide.[3]

Up to winter 2021-2022, the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) virus, causing coronavirus disease 2019 (COVID-19), has globally infected 355 million people, and the death toll has exceeded 5.6 million.[4] COVID-19 is characterized by systemic inflammation and excessive cytokine storm.[5] Possible molecular mechanisms involved in COVID-19 pathogenesis are still not fully understood.[6] Some studies showed the potential of microRNAs (miRs) for the screening, prognosis, treatment, and outcome evaluation of COVID-19. MiRs are 18-25 nucleotide long, single-stranded non-coding RNA molecules that play a role in the post-transcriptional regulation of gene expression at the RNA level by promoting messenger RNA (mRNA) degradation or translational repression.[7] Compared to other RNA molecules, miRs showed a high level of stability in the blood and resistance to chemical or enzymatic degradation.[8] MiR-155 is transcribed from the B-cell integration cluster locus located on chromosome 21 and is processed to produce functional mature 24-nucleotide miR-155.[3] So far, about 140 target mRNAs are identified for miR-155, and multiple biological processes (e.g., hematopoiesis, immunity, inflammation, and oncogenesis) are regulated by miR-155.[9] The expression of miR-155 is regulated by inflammatory cytokines and immunologic stimuli.[9] Notably, there is a potential binding site for miR-155 on the SARS-CoV-2 genome.[10] In the present study, we evaluated the expression levels of circulatory miR-155 in the sera of patients with DLBCL and/or COVID-19.

Materials and Methods

A cross-sectional study was conducted from July to December 2020 to evaluate the expression levels of miR-155 in adult patients diagnosed with DLBCL and/or COVID-19. Blood samples of 121 patients were collected from various histopathology laboratories in Tehran, Iran. Based on histopathology reports and the polymerase chain reaction (PCR) results of the SARS-CoV-2 genome, the samples were categorized into four groups, namely healthy individuals (without cancer and autoimmune/inflammatory diseases), DLBCL (histopathologically confirmed), COVID-19 (confirmed by a PCR test), and DLBCL+COVID-19 groups. The exclusion criteria were individuals under the age of 18 years; history of other malignancies, viral infections, or autoimmune/inflammatory diseases; undergoing cancer treatment (chemotherapy, radiotherapy, or gene therapy); and unsuitable blood specimen for testing (inappropriate volume or quality). Although all histopathology laboratories followed standard protocols and ethical guidelines, to avoid ethical or legal issues, 14 samples were excluded due to the missing informed consent from the patients. From the remaining 107 samples, 15 were excluded due to failure in sample RNA extraction, miR-155 primer set-up, cDNA synthesis, or real-time PCR. Eventually, 92 samples were used in the final analysis (figure 1).

Whole blood samples were delivered in tubes containing ethylenediaminetetraacetic acid (EDTA). A small portion of each whole blood sample (5 mL) was centrifuged at 3,000 rpm for 20 min at room temperature (22 °C). Then, the buffy coat was separated using a disposable sterile Pasteur pipette (Paliz Aryan Tajhiz, Tehran, Iran), aliquoted in a 200 µL microtube (Eppendorf GmbH, Hamburg, Germany), and stored at -70 °C until use.

RNA Extraction

RNX-Plus reagent (CinnaGen, Tehran, Iran), a guanidine/phenol solution for total RNA isolation, was added to a microtube containing a buffy coat and mixed by vortexing for one min. The mixture was then allowed to stand at room temperature for three min. For phase separation, chloroform (200 µL) was added to the microtube. The microtube was shaken for one min and kept at 4 °C for six min, followed by centrifugation at 13,000 rpm for 20 min at 4 °C. Then, the RNA-rich supernatant was carefully separated and aliquoted into other microtubes. An equal volume of 100% ethanol was then added to precipitate the RNA at -20 °C overnight. Next, the content of the microtube was centrifuged at 13,000 rpm at 4 °C for 40 min. After centrifugation, the supernatant was disposed, and the pellet was resuspended by adding 70% ethanol (1 mL) using vortex mixing. The mixture was centrifuged at 13,000 rpm at 4 °C for 15 min. Then, the supernatant was disposed, and the pellet was air-dried for 15 min. Finally, the RNA pellet was resuspended in DNase-/RNase-free water (30–50 µL) and stored at -70 °C until use.

The concentration of the extracted RNA was determined by optical absorbance at 260 nm using an ND-1000 UV-VIS spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). All samples showed distinct 28s and 18s ribosomal RNA bands in agarose gel (1.2% agarose with ethidium bromide) electrophoresis, indicating the quality and integrity of the eluted RNA.

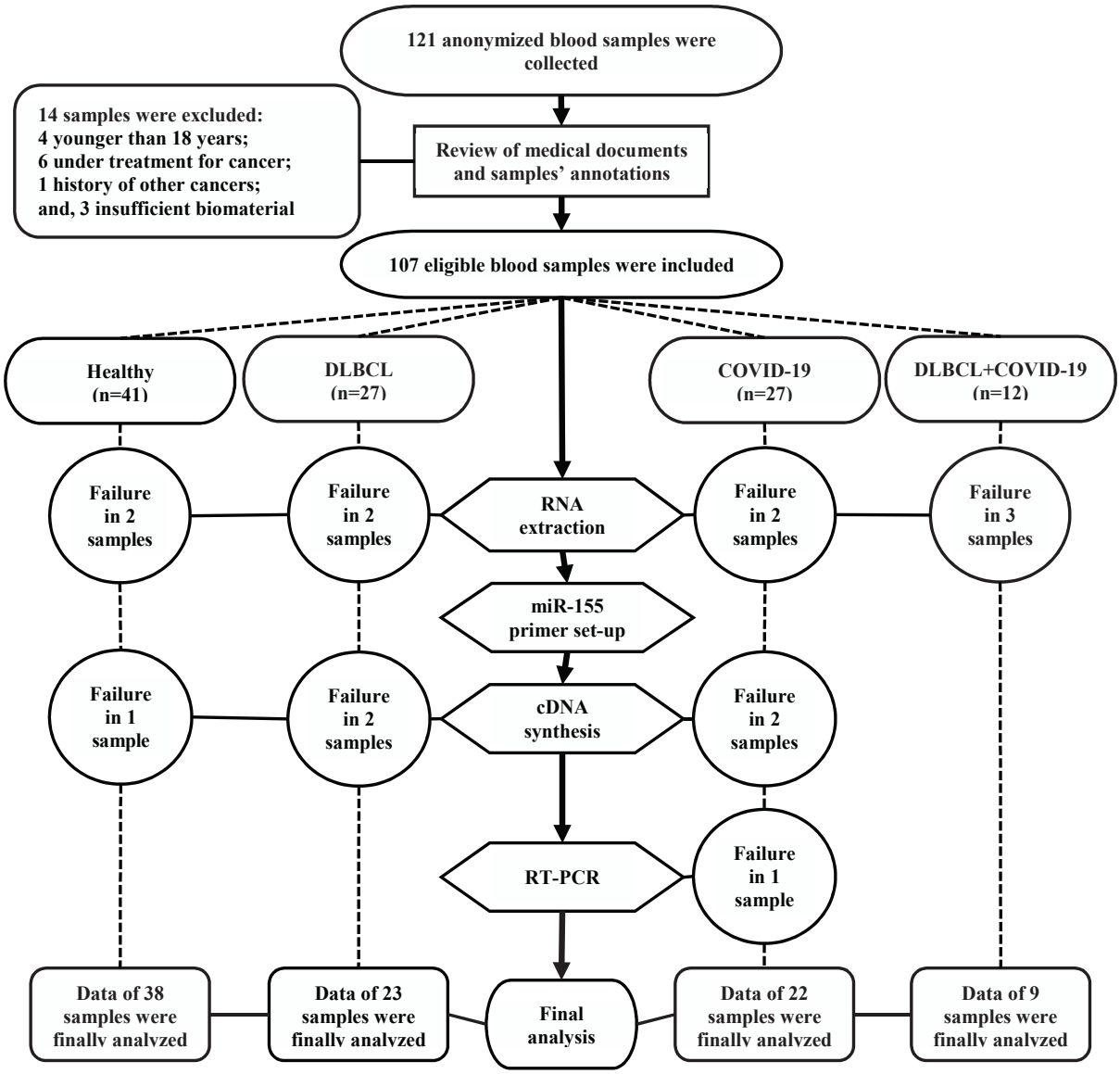

Figure 1: Of 121 collected samples, the data of 92 individuals in four study groups were analyzed. cDNA: Complementary DNA; COVID-19: Coronavirus disease 2019; DLBCL: Diffuse large B-cell lymphoma; MiR-155: MicroRNA-155; RT-PCR: Real-time polymerase chain reaction

Primer Set-up

A stem-loop forward primer was designed for miR-155 gene amplification. An original sequence was used in accordance with a previous study.[11] Nucleotides were added to elongate the loop. Some base pairs of the original stem were substituted in order to optimize the melting temperature. Nucleotides were also added to the 5' end to make a more specific primer. The Mfold web server[12] and Gene Runner software version 6.0 (Hastings Software Inc., Amarillo, TX, USA) were used for primer setup. For amplification of the U6B small nuclear RNA (RNU6B) gene, a human RNU6B miScript primer assay kit (Qiagen, Hilden, Germany) was used. Table 1 represents primer sequences used in the study.

Complementary DNA (cDNA) Synthesis

The extracted RNA was aliquoted in four microtubes (1 µg in each tube). Universal reverse primer, RNU6B forward primer, and miR-155 stem-loop forward primer (all 1.5 µL, and 10 pM) were added to each microtube. Then, DNase-/RNase-free water was added to the mixture to increase the whole volume to 13 µL. The microtubes were kept at 60 °C for five min to denature primer dimers and then incubated on ice for five min to enhance the binding of the primer to the RNA template. M-MLV reverse transcriptase (1 µL), dNTP (2 µL), and Fermentas 5× reaction buffer (all manufactured by Fermentas, Thermo Fisher Scientific, Waltham, MA, USA) were added to each microtube. The reaction was performed

Table 1: Primer sequences used for the amplification of microRNA-155 and RNU6B genes

Primer	Sequence (5'–3')
Hsa-mir-155 forward[a]	TGCTAATCGTGATAGGGG
MiR-155 forward[b]	GTCGTATGCAGAGCAGGGTCCGAGGTATTCGCACTGCATACGACAACCCCT
MiR-155 forward[c]	GAGGATGTATAATCGTAATTGT
RNU6B forward	CGGTTTAATGCTAATCGTGA
Universal reverse	GAGCAGGGTCCGAGGT

Hsa-miR: Human microRNA; MiR-155: MicroRNA-155; RNU6B: U6B small nuclear RNA; [a]Original primer sequence is used from the literature. [b]Utilized for a real-time polymerase chain reaction. [c]Utilized for cDNA synthesis, the primer has a stem-loop structure.

according to the manufacturer's instruction using the following conditions: at 25 °C for 15 min, at 37 °C for 10 min, at 42 °C for 45 min, and at 75 °C for 10 min. Then, the microtubes were stored at -20 °C until use.

Polymerase Chain Reaction

Real-time PCR analysis was carried out on Rotor-Gene 6000 HRM real-time PCR machine (Corbett, Mortlake, Australia) using an SYBR® Premix Ex Taq™ PCR kit (Takara Bio Inc., Kusatsu, Shiga, Japan). RNU6B and miR-155 forward primers, universal reverse primer (all 0.5 µL and 10 pM), plus RNU6B and miR-155 cDNA templates (both 2 µL) were added to the microtube, and deionized water was then added to increase the whole volume to 25 µL. According to the manufacturer's instruction, the following thermocycling conditions were applied: polymerase activation at 95 °C for 15 seconds, followed by 45 cycles of denaturation at 94 °C for 15 sec, annealing at 55 °C for 30 sec, and extension at 65 °C for 35 sec.

Melting curve analysis was performed between 55 °C and 95 °C with increments of 0.5 °C every three sec. No-template reaction was included as a negative control. The RNU6B gene was used as an endogenous control. Relative quantification of miR-155 gene expression was determined from cycle threshold values (Ct) for miR-155 and RNU6B genes. Then, the ΔCt was calculated by subtracting the Ct values of RNU6B from the Ct values of miR-155.

Statistical Analysis

Descriptive statistics (frequency, mean, standard deviation, and standard error) were used to report the baseline findings. Pearson's correlation was used to assess the associations between miR-155 relative expression (-ΔCt) and age, followed by independent samples *t* test to compare -ΔCt between men and women in the groups. Fisher's exact test was used to assess sex distribution in the groups, and one-way analysis of variance (ANOVA) and Fisher's least significant difference *post hoc* test to compare the mean age and -ΔCt between the groups. ANOVA and *t* test assumptions were checked using Kolmogorov-Smirnov for normality and Levene's test for homogeneity of variance (not reported). Significant differences of -ΔCt in the study groups were evaluated using univariate ANOVA adjusted for age and sex. The Bonferroni correction was used for pairwise comparisons. The level of significance was 0.05. All analyses were conducted using SPSS software, version 25.0 (IBM Corp., Armonk, NY, USA).

Ethical Considerations

The study was approved by the Ethics Committee of Jiroft University of Medical Sciences, Kerman, Iran (IR.JMU.REC.1400.014). The study was conducted in accordance with the ethical principles of the Declaration of Helsinki, including anonymization of blood samples, and study conduct based on ethical recommendations and standard protocols. All participants had given informed consent for the use of their blood samples and data for research purposes.

Results

Of the 92 samples included in the final analysis, 38 were from healthy individuals, nine patients were confirmed cases of both DLBCL and COVID-19, 23 had DLBCL, and 22 had COVID-19. The majority of blood samples (64.1%) were from male patients. The frequency distribution of sex across the study groups was not statistically different (P=0.834). Tables 2 and 3 present demographic characteristics and Ct values of the four study groups. As shown in figure 2, the highest expression of miR-155 was observed in patients with DLBCL+COVID-19 (-ΔCt: 4.33, 95% confidence interval: 2.82–5.83). There was no significant correlation between demographic characteristics (sex and age) and miR-155 expression in the study groups. The coefficients of Pearson's correlation between age (one-year increments) and miR-155 expression in the DLBCL, COVID-19, and DLBCL+COVID-19 groups were 0.007 (P=0.755), 0.006 (P=0.500), and -0.050 (P=0.187), respectively.

Table 2: Demographic characteristics of the four study groups

Variable		Healthy	DLBCL	COVID-19	DLBCL+COVID-19	P value
Sex (n, %)	Women	12 (31.6)	8 (34.8)	9 (40.9)	4 (44.4)	0.834*
	Men	26 (68.4)	15 (65.2)	13 (59.1)	5 (55.6)	
Age (years, mean±SD)		57.2±9.5[A]	60.6±7.7[B]	48.2±15.9[C]	58.1±18.9[D]	0.006†

COVID-19: Coronavirus disease 2019; DLBCL: Diffuse large B-cell lymphoma; *Fisher's exact test, level of significance is 0.05. †One-way analysis of variance, level of significance is 0.05. Fisher's least significant difference post-hoc test: A=B (P=0.290), A=D (P=0.855), B=D (P=0.589), C<A (P=0.007), C<B (P=0.001), C<D (P=0.043)

Table 3: Cycle threshold values of microRNA-155 and RNU6B in the four study sample groups. Data expressed as mean and standard deviation

	Healthy	DLBCL	COVID-19	DLBCL+COVID-19	P value*
Ct_{RNU6B}	18.48±5.68[A]	22.97±6.14[B]	21.98±6.76[C]	26.32±7.30[D]	0.002
$Ct_{miR-155}$	20.40±6.12	20.71±5.85	20.03±6.69	21.99±6.28	0.879

Ct: Cycle threshold value; COVID-19: Coronavirus disease 2019; DLBCL: Diffuse large B-cell lymphoma; MiR-155: MicroRNA-155; RNU6B: U6B small nuclear RNA gene; *One-way analysis of variance, level of significance is 0.05. Fisher's least significant difference post-hoc test: A<B (P=0.008), A<C (P=0.038), A<D (P=0.001), B=C (P=0.596), B=D (P=0.175), C=D (P=0.082)

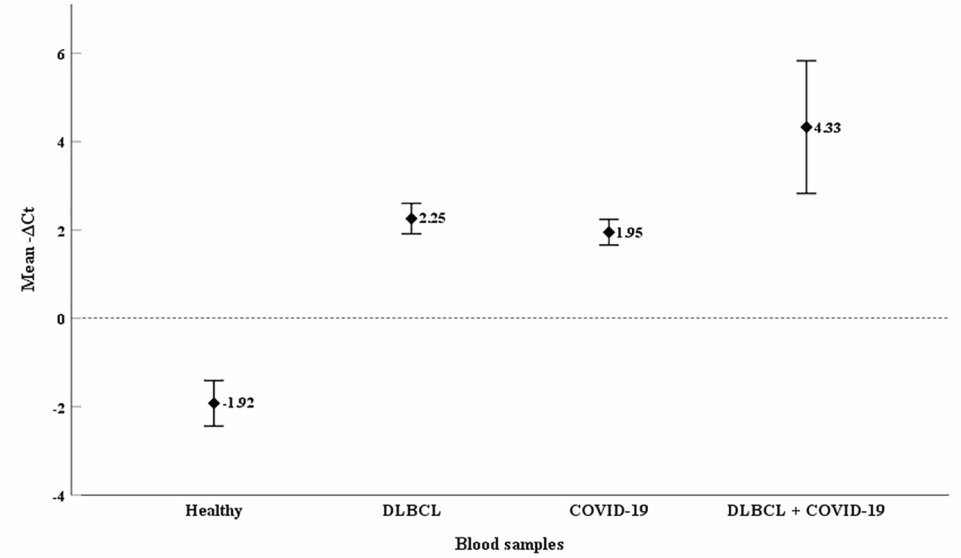

Figure 2: Relative expression of microRNA-155 increased in the patient groups. Numbers and bars respectively represent the mean, –ΔCt, and 95% confidence interval of the mean. Ct: Cycle threshold value; COVID-19: Coronavirus disease 2019; DLBCL: Diffuse large B-cell lymphoma

The relative expression of miR-155 (mean±SEM) was not different between men and women in the healthy (-1.94±0.27 and -1.88±0.56, respectively, P=0.916), DLBCL (2.33±0.20 and 2.11±0.29, respectively, P=0.528), COVID-19 (2.13±0.18 and 1.68±0.18, respectively, P=0.115), and DLBCL+COVID-19 (4.66±0.64 and 3.91±1.32, respectively, P=0.602) groups.

The expression of miR-155 in the DLBCL (mean difference of -ΔCt: 4.18, P<0.001) and COVID-19 (3.87, P<0.001) groups was significantly higher than those in the healthy group. Patients suffering from both DLBCL and COVID-19 also showed a significantly higher expression than the healthy individuals (6.25, P<0.001). The expression of miR-155 in the DLBCL and COVID-19 groups was statistically the same (-0.30, P=0.422). However, the expression of miR-155 in the DLBCL+COVID-19 group was higher than that of the DLBCL (2.07, P<0.001) and COVID-19 (2.37, P<0.001) groups (table 4).

Discussion

In line with a previous study,[13] we found that the expression of miR-155 is not associated with sex or age. This finding greatly simplifies the process of population screening or individual patient follow-up (repeated measurements, disease monitoring, and treatment follow-up). The expression of miR-155 in serum could be utilized as an alternative approach for screening and diagnosis, when a gold standard (e.g., histopathology) is not available or inapplicable. This evaluation method is non-invasive, safe,

Table 4: Comparison of microRNA-155 relative expression between study sample groups based on the mean differences of -ΔCt (calculated by subtracting the Ct values of RNU6B from the Ct values of microRNA-155)

Variable	Mean difference of -ΔCt (95% CI)*,a					
	DLBCL	P value	COVID-19	P value	DLBCL+COVID-19	P value
Healthy	4.18 (3.51-4.85)	<0.001	3.87 (3.19-4.55)	<0.001	6.25 (5.31-7.19)	<0.001
DLBCL	-	-	-0.30 (-1.06-0.45)[b]	0.422	2.07 (1.07-3.07)	<0.001
COVID-19	-	-	-	-	2.37 (1.37-3.38)	<0.001

CI: Confidence interval; Ct: Cycle threshold value; COVID-19: Coronavirus disease 2019; DLBCL: Diffuse large B-cell lymphoma; RNU6B: U6B small nuclear RNA gene; *One-way analysis of variance, Fisher's least significant difference post-hoc test. [a]Numbers are calculated by subtracting $-\Delta Ct_{row}$ from $-\Delta Ct_{column}$. [b]The difference was not significant (P=0.422), all other observations were significant (P<0.001)

and inexpensive compared to diagnostic biopsy or aspiration for specimen collection.[14]

Regulation of miR-155 plays a pivotal role in linking inflammation with malignancy.[15, 16] In line with previous studies, we found that miR-155 is highly expressed in DLBCL, shown to decelerate cell apoptosis, and induce cell proliferation, migration, and invasion.[17, 18] Inflammatory cytokines and immunologic stimuli activate nuclear factor-kappa B, which in turn induces miR-155 expression.[19, 20] Increased expressions of miR-155 down regulates proapoptotic proteins, suppressor of cytokine signaling protein 3 (SOCS3), B-cell lymphoma protein 6 (BCL6), histone deacetylase 4 (HDAC4), and Src homology 2-containing inositol 5′ phosphatase 1 (SHIP1). Consequently, it increases the survival and proliferation of B-cells and makes cells evade apoptosis.[20-25] It is also suggested that increased miR-155 up regulates cell cycle proteins and induces B-cell proliferation.[22, 26]

In line with previous studies, we observed an increased expression of miR-155 in COVID-19 patients.[27, 28] Previously reported progression from mild to severe COVID-19, and conversely, alleviation of inflammation and virus-induced cytokine storm might be associated with down regulation of miR-155.[28, 29] On the other hand, bioinformatics analyses have shown that increased miR-155 up regulates the expression of angiotensin-converting enzyme 2, an essential SARS-CoV-2 entry receptor, in oral mucosae.[30] According to the literature, miR-155 can protect or damage tissue during the inflammatory process depending on the disease trigger.[31]

In viral infections, miR-155 regulates the immune response and enhances antiviral T-cell responses through the type I interferon signaling pathway.[10, 32] Increased expression of miR-155 induces tissue inflammation through indirect up regulation of pro-inflammatory cytokines (e.g., tumor necrosis factor α, interleukin-1β, T-cell differentiation) and activation of macrophages and antigen-specific helper T-cells (Th1 and Th17).[33-35] It is also suggested that miR-155 plays a role in the negative feedback loop in toll-like receptor and interleukin-1 pathway, and down regulates the production of inflammatory cytokines and matrix metalloproteinases (MMP1 and MMP3).[36] Therefore, miR-155 is possibly expressed to modulate the inflammation process and control inflammatory tissue damage. In addition, miR-155 positively regulates antibody-mediated signaling in B-cells. In response to inflammatory signals, B-cells increase the expression of miR-155, which in turn down regulates purine-rich box-1 (PU.1) transcription factor and paired box protein 5, leading to terminal cell differentiation and antibody production.[37, 38] On the other hand, decreased expression of PU.1 also reduces CD10 protein in B-cells, resulting in a more aggressive form and poor prognosis of DLBCL.[19] Hence, an increased miR-155 expression in the event of comorbidity with COVID-19 may worsen the prognosis of DLBCL patients.

The main limitation of the study, due to limited financial resources, relates to the manual processing of some procedures rather than the use of commercially available kits. This may have led to the loss of samples and the failure of some procedures. The miR-155 expression was relatively quantified in our study. Given high stability and low variability in the expression levels of the RNU6B gene, we utilized this housekeeping gene as an endogenous control to normalize the expression of the miR-155 gene. We studied samples from different histopathology laboratories. However, there is a potential bias emanating from inter-rater variability despite all samples being collected, prepared, and examined in accordance with standard protocols. Another limitation was the fact that the study groups were not age-matched, although we adjusted the results by age and sex. The results of the regression analysis also did not show any association between age and sex, or their association with the expression of miR-155 (not reported). Finally, limitations associated with the descriptive design of our study might have affected the reliability and validity of our results. Given the low sample size, further

studies with a larger sample size are required to assess the correlation between miR-155 expression and clinical presentation, severity, and grade of the DLBCL and COVID-19.

Conclusion

The expression of miR-155 increased in patients with DLBCL and/or COVID-19 compared to healthy individuals. Further molecular investigations and clinical trials are recommended to confirm the diagnostic, prognostic, and therapeutic application of miR-155 in these patients.

Acknowledgment

The present manuscript was extracted from the PhD dissertation by SR Mahdavi-Anari. The authors would like to thank the donors and laboratories for providing the blood samples.

Authors' Contribution

B.K: Concept and study design; S.R.M.A: concept and study design, performing laboratory procedures, analyzing and interpretation of the data; drafting; K.A: Statistical advice on study design and data analysis; F.R: Statistical advice on study design and data analysis, analyzing and interpretation of the data, drafting. All authors collaborated in data acquisition. All authors critically reviewed the manuscript. All authors approved the present version of the manuscript. All authors agreed on being accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Conflict of Interest: None declared.

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