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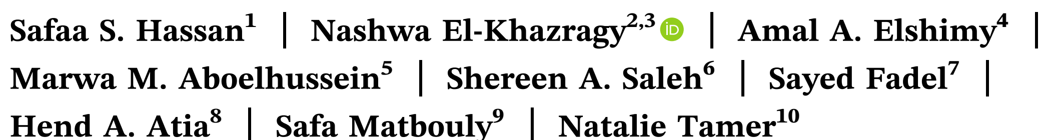
RESEARCH ARTICLE

In vitro knock-out of miR-155 suppresses leukemic and HCV virus loads in pediatric HCV-4–associated acute lymphoid leukemia: A promising target therapy



Safaa S. Hassan[1] | Nashwa El-Khazragy[2,3] | Amal A. Elshimy[4] | Marwa M. Aboelhussein[5] | Shereen A. Saleh[6] | Sayed Fadel[7] | Hend A. Atia[8] | Safa Matbouly[9] | Natalie Tamer[10]

[1]Clinical Pathology Department, National Cancer Institute, Cairo University, Cairo, Egypt
[2]Clinical Pathology/Hematology and Biomedical Research Departments, Faculty of Medicine, Ain Shams University, Cairo, Egypt
[3]Biomedical Research Department, Global Research Labs, Cairo, Egypt
[4]Medical Microbiology and Immunology Department, Faculty of Medicine, Cairo University and New Giza University, Cairo, Egypt
[5]Medical Biochemistry and Molecular Biology Department, Faculty of Medicine, Ain- Shams University, Cairo, Egypt
[6]Internal Medicine Department, Faculty of Medicine, Ain- Shams University, Cairo, Egypt
[7]Pediatric Oncology Department, National Cancer Institute, Cairo University, Giza, Egypt
[8]Clinical Pathology Department, Faculty of Medicine, New Giza University, Giza, Egypt
[9]Department of Pediatrics, Faculty of Medicine, Ain Shams University, Cairo, Egypt
[10]Faculty of Biotechnology, October University for Modern Sciences and Arts (MSA), Giza, Egypt

Correspondence
Nashwa N. El-Khazragy, Professor of Clinical Pathology/Hematology and Biomedical Research Departments, Faculty of Medicine, Ain Shams University, Cairo, Egypt and Managing Director of Biomedical Research Department, Global Research Labs, Medical Center, Nasr, Cairo, 11787, Egypt, P.O. box 11381.
Email: nashwaelkhazragy@med.asu.edu.eg and nashwa@globalresearchlabs.com

Abstract

Hepatitis C virus (HCV) infection is a major public health problem, having a high prevalence in Egypt. Leukemia and lymphoma have been associated with HCV infection. MicroRNA-155 (miR-155) has been reported to play a regulatory role in cancer, inflammation, and immune response to infection. The expression level of miR-155 in HCV viremic patients is controversial; although high miR-155 levels were demonstrated in HCV genotypes 1,2, and 3, low levels of miR-155 were detected in Egyptian patients with HCV genotype 4. Several studies have investigated the correlation between the levels of miRNA-155 and the replication of HCV, others have evaluated miRNA-155 as a prognostic biomarker in different types of cancer. No studies have investigated the impact of miRNA-155 knockdown on HCV pediatric patients associated with childhood acute lymphoblastic leukemia (ALL). We knocked-out the miR_155a in cultured polymorphonuclear cells (PBMCs) obtained from 60 children with ALL; 30 were associated with HCV-4 infection and 30 were HCV negative. The miR_155a, HCV viral load, and cell proliferation werre assessed in treated and untreated cells using TaqMan assay quantitative polymerase chain reaction. We found that miRNA-155 was significantly upregulated by seven folds in the HCV-4 associated ALL group; while being linked to high HCV viral load and leukemic burden, miR_155a knock-out can improve the disease outcome. We conclude that miR-155 is a critical miRNA that is considered a therapeutic target in pediatric HCV leukemic patients.

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KEYWORDS
HCV associated leukemia/lymphoma, in vitro knock-down, miR-155, pediatric, prognosis

1 | INTRODUCTION

MicroRNAs (miRNAs) are small noncoding RNAs. They are epigenetic regulators that target specific cellular messenger RNA (mRNA) to regulate its gene expression by altering its transcription process. miRNAs are involved in a very wide scale of physiological and pathological processes.[1] Their role has been demonstrated in Yin and Yang study.[2] Physiologi- Physiologically, it is involved in hematopoiesis, immunity, and inflammatory processes.[3] Also, it is involved in the pathogenesis of malignancies and heart and renal diseases.[4-6] miRNAs have a critical role in viral infections, and one of them is hepatitis C virus (HCV) infection. Several miRNAs have been involved in viral replication pathogenesis, such as miR-155, miR-122, miR-448, miR-199a, and miR-196.[7] Also, miRNAs play an important role in hematological malignancies.[4] Many studies have investigated the expression patterns of miRNAs in chronic lymphocytic leukemia (CLL)[8], acute myeloid leukemia (AML), and adult T-cell leukemia (ATL), and many B-cell lymphomas.[4]

HCV is one of the most common oncogenic viruses. HCV chronic infection can lead to hepatocellular carcinoma and non-Hodgkin lymphoma (NHL).[9] New emerging evidence suggests that HCV infection increases the risk of lymphoid neoplasms, with a predominance of large B cell lymphoma.[10] HCV chronic infection may induce B-cell lymphoma by sustained B cells stimulation through chronic antigenic stimulation, which will lead to increased production of cryoglobulins.[11] Also, there is strong evidence that treatment with antiviral therapy (AVT) alone without chemotherapy can repress many silent lymphomas.[12] Finally, it is supposed that viremia is a critical predictor of the carcinogenic effect of HCV infection.[9] It has been found that long-standing viremia with immunosuppression is involved in the development of NHL.[9] Treatment with highly effective AVT is recommended in HCV-infected patient with other malignancies to avoid the poor treatment response and recurrent lymphoma or leukemia.[9]

miR-155 has a critical prognostic role in HCV infection, leukemia, and lymphomas.[13] miR-155 expression is one of the discrepancies in HCV genotype 4 infections, as it does not vary in polymorph nuclear cells (PMNCs) compared to healthy controls, unlike its expression in HCV genotypes 1, 2, and 3 infections.[14] The different expression of miR-155 may affect the prognosis and may explain the resistance to the treatment pattern of HCV genotype 4.[14] On the other hand, studies that investigated the role of miR-155 in CML and AML demonstrated that it is upregulated in cells and circulating exosomes.[4,15-17] There was a negative correlation between the expression of miR-155 and the response to antiviral drugs; however, a positive correlation with disease progression has been observed.[18,19] Patients who achieved complete remission showed low levels of miR-155 in the plasma.[19] Regarding the prognosis, a high level of miR-155 is a predictor of a bad prognosis in patients with CLL[8] and AML.[20-23]

miR-155 has a clear association with lymphomas and leukemias pathogenesis, progression, and response to treatment. At the same time, it has a direct relation with the immune response to HCV infection and its replication and response to treatment as well. In our study, we will investigate the impact of miR_155a knock-out on leukemic burden and HCV_4 viral replication in pediatric patients with childhood acute lymphoblastic leukemia associated with HCV genotype 4 infections. (Figure 1).

2 | STUDY DESIGN

Peripheral blood samples were collected from 60 de-novo Acute lymphoblastic leukemic children, 30 children were positive for HCV, whereas, 30 were negative who were attending the Pediatric Hematology Unit at the National Cancer Institute, Cairo, Egypt. Written consent was obtained from the parents before sample collection The diagnosis of Leukemia was based on the analysis of bone

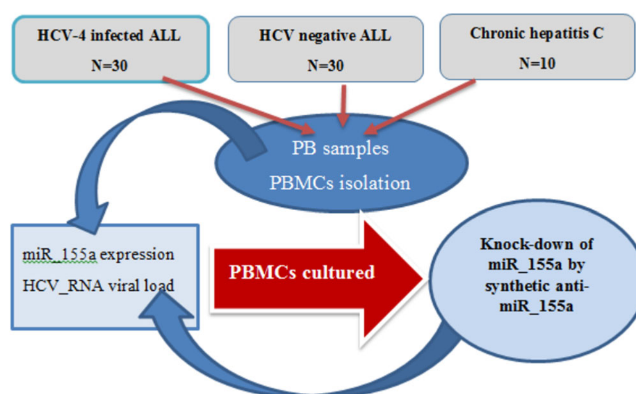

FIGURE 1 A workflow hierarchy chart illustrates the sequential steps followed in the study; PB samples were collected from three groups of children, PBMCs were separated, purified and characterized then cultured in specific conditions and finally miR_155a knocked-down in cultured cells. The miR_155a, HCV_RNA viral load and cell viability were assessed in treated and untreated cultured cells. HCV, hepatitis C virus; miR, microRNA; PBMC, polymorphonuclear cell

marrow samples by morphological, cytochemical, immune-phenotyping, and cytogenetics. Ten children who had chronic HCV infection and did not have a history of cancer or hematological disease were included as a control group. They were collected from the Virology Unit at the National liver Institute, Egypt. HCV genotype 4 infection was confirmed in both leukemic and control sera by HCV-RNA testing using the artus HCV RG reverse transcription polymerase chain reaction (RT-PCR), cat no: 4518265 (Qiagen, Hilden, Germany) according to the manufacturer's instructions (limit of detection 50 viral copies/mL or 25 IU/mL). HCV virus genotype was determined; other HCV virus genotypes were excluded from the study.

2.1 | Cell culture

Peripheral blood were drawn for cell culture in sterile centrifuge tubes containing sodium citrate. Lymphocyte cells were isolated immediately from PB samples of HCV-4-infected and HCV-negative leukemic children using lymphoprep reagents cat no: NC0665098 (Fisher Scientific, UK); the protocol followed was in accordance with the manufacturer's instructions. For lymphocyte culture, the sedimented lymphocytes were suspended in 500 uL of Eagle's minimum essential mediums (EMEM) supplemented with 10% fetal calf serum and counted by a hemocytometer. The cell suspension was diluted to a final volume of 100 mL with EMEM in nonsiliconized 500 mL Erlenmeyer flasks that contained a Teflon-coated magnetic bar and incubated at 37°C on a magnetic stirrer. The initial cell population densities were adjusted to 5×10^4, daily; the culture was fed by the addition of 100 mL EMEM media to reach an increase in volume by 100%, 50%, and 33%, respectively, during 72 hours. Every day, the total and viable cells were counted by a hemocytometer for all cultures. The cultured lymphoblast "Lymphocyte progenitors" cells were characterized by the expression of CD34 and CD10 /CD19 for B lymphocyte or CD3/CD5 for T lymphoblast.

2.2 | miR_155 inhibition assay

The miRNA-155 gene expression is inhibited in cultured lymphocyte progenitors cells to observe, measure, and determine the efficiency of miRNA-155 interference on the elimination of their growth. The cell count was adjusted to $0.4\text{-}1.6\times10^4$ cells and transfected with anti-has-miR-155-5p miScript miRNA inhibitor were seeded per well in a 96-well plate, 100μL of an appropriate culture medium containing serum and antibiotics were added. A triplicate of 1.5 μL anti-hsa-miR-155-5p miScript miRNA Inhibitor (20 μM stock) in 100 μL culture medium without serum "this will give a final miRNA final miRNA inhibitor concentration of 50 nM after adding complexes to cells," then 3 μL of Hiperfect transfection reagent were added to a diluted miRNA and well mixed by vortexing. The samples were incubated for 5 to 10 minutes at (15-25, "room temperature") for cells to form a transfectant complex. The complexes were added dope-wise onto the cells, and the plate was gently swirled to ensure the uniform distribution of transfection complexes. The cells with the transfection complexes with their normal growth conditions were incubated at 37°C and 5% CO_2.

2.3 | Cell cytotoxicity assay

The cell cytotoxicity assay was performed using the CellTiter 96 solution (Promega, Germany), A 20μL of CellTiter 96 AQ_{ueous} One Solution Reagent were added into each well of the 96-well assay plate containing 100μL of culture medium., the plate was incubated in 37°C for 1 to 4 hours in humidified, 5% CO_2 atmosphere and the conversion of 3-(4, 5-dimethylthiazol-3-yl)-2,5-diphenyl tetrazolium bromide to blue formazan was measured by reading the absorbance at wavelength of 490 nm using 96-well plate reader.

2.4 | Total RNA extraction and purification

Total RNA was extracted from harvested cells using the *mir*Vana PARIS kit (Ambion); extraction was performed according to the manufacturer's instructions. The extracted RNA was eluted in 100 μL elution buffer, and RNA integrity was confirmed by measuring the optical density at 260/280 nm using a NanoDrop Spectrophotometer (Thermo Fisher scientific). The extracted RNA is stored at −80°C until use.

2.5 | Quantitative real-time PCR

The expression level of miRNA-155 was measured using specific TaqMan MicroRNA Assays (Applied Biosystems) for miR-16 (reference miRNA) and miR-155. The PCR reaction was performed as described by the manufacturer. Briefly, 100 ng of total RNA was reverse-transcribed using primers specific to each miRNA target followed by real-time PCR on a StepOne Real-Time PCR System using TaqMan miRNA primers and probes (Applied Biosystems). Triplicate samples, validated endogenous controls, and interassay controls were used throughout. The miRNA expression levels were calculated by the ΔC_t method: $\Delta C_t =$ mean value C_t (reference miR-16)−mean value C_t (miRNA of interest). The relative expression of miRNA of interest corresponded to the copies of gene expression/$2^{\Delta C_t}$ value.

2.6 | Statistical analysis

Statistical analyses were performed using the SPSS software package, version 22.0. The potential association between the miR-155 concentration in cultured cells with HCV viral load and leukemic blasts percentage was identified using the χ^2 or two-tailed Fischer's exact test. For nonparametric comparisons, univariate analyses of the Mann Whitney-*U* test of two independent variables were used. Parametric data were presented as mean ± and nonparametric as median and interquartile range. $P < .05$ in all cases was considered statistically significant.

3 | RESULTS

3.1 | miR_155a expression is upregulated in samples from ALL children

The miR-155a expression was measured in cultured PMNC samples obtained from PB samples of HCV-positive and negative acute lymphoblastic leukemia using quantitative PCR. The expression level was normalized to HCV-positive polymorphonuclear leucocytes (PMNLs) collected from children with chronic HCV infection. The results revealed that all cultured PMNLs of ALL patients expressed higher levels of miR_155a regardless they were HCV-infected or not. The expression level of miR-155 was about seven folds higher in the HCV-4 infected ALL cells vs the HCV-4 negative cells. Data were presented in a boxplot graph Figure 2A. The log median of miR-155 expression in the HCV-4 infected leukemic group with 95% confidence interval was 47.8 (12.5-168) while in the HCV-4 negative, it was 6.2 (2.8-19) with a *P* value .001.

3.2 | miR_155a is upregulated in HCV-4 infected PBMC

To investigate the link between HCV_4 infection and miR_155a expression, PMNLs from children with chronic HCV-4 infection was cultivated and then miR-155a expression was analyzed and compared to uninfected PBMC obtained from two healthy donors. A significant difference was obtained between both groups; the HCV-4-infected PMNLs cells showed increase in miR-155a expression by three folds in infected cells compared to uninfected PBMC, Figure 2A. Moreover, the miR_155a expression was positively correlated with HCV-RNA viral load; higher expression levels were significantly associated with high viremia (Figure 2B).

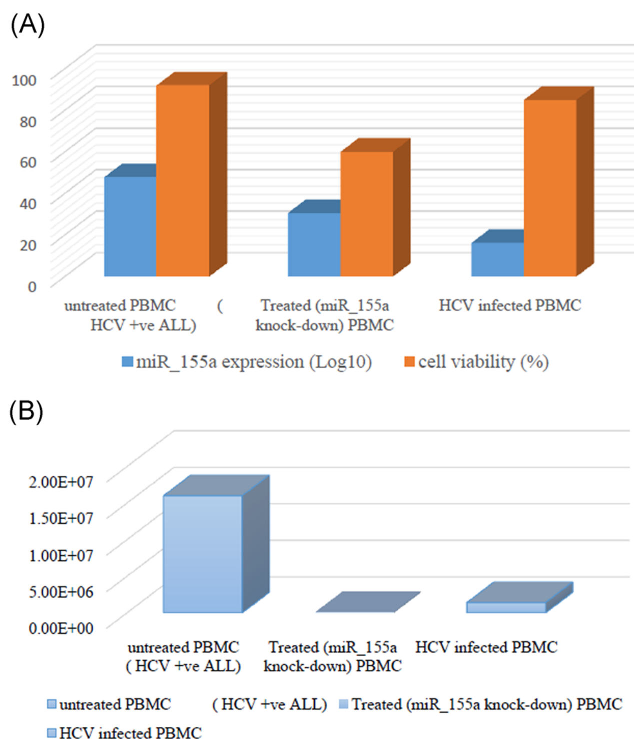

FIGURE 2 Expression of miR_155a in HCV-4 infected ALL samples, HCV-4 infected PMNLs and miR_155a knocked-out HCV-4 infected ALL cells. miR_155a was detected by TaqMan Probe based qPCR, the detected values in PBMC sample obtained from healthy donors were used as a calibrator. A: shows miR-155a expression levels in tested cell lines, blue bars represent the mean values of miR_155a expression (47.8,30.6 and 16.2) detected in the treated and untreated HCV-4 infected ALL cells as well as HCV-4 infected samples; respectively. Moreover; the red bars represent the cell viability in the tested cell lines, higher miR_155a level is associated with high proliferation and increase cell viability, however; a marked reduction in cell viability was observed in HCV-4 infected ALL cells that were transfected by anti-miR_155a. Whereas, the percentage of cell viability was 92% and 85% in HCV-4 infected ALL cells and HCV-4 infected samples, a significant reduction of cell viability (62%) was determined in HCV-4 infected ALL cells that was treated by anti-miR_155a. B: represents the mean values of HCV-RNA viral load (copies/μL) in the three tested cultured cells. A significant decline in HCV-RNA viral load was detected in miR_155 knocked-out HCV-4 infected ALL cells (1.5×10^5) compared to (1.6×10^7) and (1.4×10^6) in untreated and HCV-4 infected cells; respectively. HCV, hepatitis C virus; miR, microRNA; PMNLs, Polymorphonuclear leucocytes; qPCR, quantitative polymerase chain reaction

3.3 | Effects of miR_155a inhibition in HCV-4 infected ALL cells

To verify the effect of miR_155a inhibition on leukemic cell proliferation and viral replication in HCV_4-associated ALL, the cultivated PMNLs were transfected with a synthetic miR_155a inhibitor. qPCR results revealed that functional knock-out of miR_155a

results in decrease cell viability up to 62% and reduction in HCV-RNA viral load by two folds compared to nontransfected cells. These observations provide evidence that knock-out of miR-155a favors the control of leukemic proliferation and viral replication in HCV-4-associated acute lymphoblastic leukemia cells. In addition, lower miR_155a expression levels were significantly associated with the lower proliferation rate of leukemic cells and low HCV viremia ($P < .001$).

4 | DISCUSSION

Pediatric leukemia is one of the most aggressive types of malignancies in childhood. It carries a poor prognosis, especially if it is associated with HCV infection; therefore, we are searching for prognostic and therapeutic biomarkers as an attempt to improve disease outcome. miR-155 is an oncomiR that is highly expressed in different types of leukemia and lymphoma.[4,15] In our study, we found very high levels of miR-155, about seven folds, in HCV_4-associated ALL in pediatric patients in comparison with the HCV negative leukemic group; moreover, two folds increase in the expression was detected in HCV-negative leukemic patients in contrast to children with HCV_4 chronic infection. This big difference in the level of expression denotes the oncogenic effect of miR-155 as well as its impact on HCV_4 viral load. Regarding prognosis, miR-155 may have a good clue in these patients. Our results show there is a significant difference in levels of miR-155 in patients with high leukemic burden and high viremia; miR-155 was more than the double in patients with blast count exceeding 70% and HCV_RNA viral load greater than 100 000. So, we may judge the aggressiveness of the disease depending on the level of miR-155 expression. Although there was no any significant correlation between miR-155 expression and other laboratory investigations including liver function tests and lactate dehydrogenase “data are not presented”.

miR-155 expression was significantly elevated in patients with HCV-4 associated ALL cultured cells. At the same time, the expression level of miR-155 in PMNLs which were knocked-out by synthetic miR_155a inhibitor was significantly decreases than in uninhibited cells. In addition, HCV-RNA viral load was significantly declined in miR_155a knocked-out cells compared to untreated cells. These observations suggested the correlation between elevated levels of miR_155a expression and the survival of leukemic blasts and HCV. Accordingly, it highlights the therapeutic value of miR-155a knock down in improving the disease phenotype of acute leukemia associated with HCV-4 infection.

Recent studies pointed to the role of miR-155 in changing the output of bone marrow hematopoiesis.[3] During the inflammatory response, there are changes in the bone marrow itself with deviation toward myeloid differentiation line with suppression in lymphoid and erythroid lines.[3] It has been found that this process is mediated through many mechanisms like[1] interferon signaling–mediated hematopoietic stem cell proliferation.[2] Activation of TLR4 with activation of MyD88 signaling results in enhanced myeloid differentiation[3] nuclear factor-κB (NF-κB) signaling pathway.[3,24,25] The overexpression of miR-155 initiates a myeloproliferative disorder. Increasing research points to NF-κB activation in human AML CD34+ cells and many other myeloid malignancies. Our study supports the theory that inflammatory hematopoiesis pathways may have a critical role in lymphoid malignancies, as it is constitutive in these cases with, no doubt, miRNAs; especial,ly miR-155 is part of these disruptions. So, we think high levels of miR-155 in the presence of HCV infection with leukemia may increase the aggressiveness of leukemia, worsen the response to treatment, and may induce more hematological malignancies.

We suggest that miR-155 may be a vital target in patients with both HCV-chronic infection and leukemia. miR-155 plays a role in the viral presence and increases its replication.[7] Targeting miR-155 may suppress HCV replication. Lowering the viral load has a great impact on the immune system; lowering the viral load will decrease immune system sustained stimulation and lower the risk of developing B-cell lymphoma. Also, targeting miR-155 may have a beneficial effect on leukemia itself. After explaining the possible oncogenic effect of miR-155, our study also found that complete remission is associated with low level of miR-155 expression.

If we talked about the strong points in our study, to the best of our knowledge, this is the first study to investigate the therapeutic potential of miR-155 in leukemic children with chronic HCV infection. There are many studies illustrating the role of miR-155 in HCV infection and leukemia, separately. But we also have some limitations: we did not study the mechanistic pathways by which miR_155a inhibition suppresses leukemic cell proliferation and HCV replication in pediatric ALL.[10] We suggest more research to investigate the roles which miR-155 play in those patients. Also, we recommend further studies that can target miR-155 and clarify the true effect of suppressing miR-155 in this condition.

In conclusion, miR-155 is a critical miRNA that is considered a potential therapeutic target in children with HCV-4 associated ALL. Expression of miR-155 is upregulated in HCV-4 positive leukemic patients

versus HCV-4 negative children. It is also upregulated in chronic HCV patients vs healthy donors. In addition;, the knock-out of miR_155a could suppress the leukemic burden and HCV viremia, thus improving patients outcome. We recommend further studies that target miR-155 as a potential regulator in leukemic children with HCV chronic infection.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

El-Khazragy N conceived the presented idea, developed the theory, performed the computations, and verified the analytical methods. Elshimy A, Shawky S, Atia H and Aboelhussien M investigated and supervised the findings of this study. All authors discussed the results and contributed to the final manuscript. Matboly S, Saleh S and Fadel S helped in samples and patients data collection as well as clinical interpretations of results. El-Khazragy N wrote the manuscript with support from Elshiemy, who helped supervise the project. Matbouly S, Tamer N and Atia H carried out the experimental methods, developed the theoretical formalism, performed the analytic calculations, and performed the numerical simulations. El-Khazragy contributed to the final version of the manuscript and supervised the project.

ORCID

Nashwa El-Khazragy http://orcid.org/0000-0001-6646-4674

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