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ROLE OF MIR-155 GENE SILENCING IN CONTROL ONCOGENESIS IN ACUTE MYELOID LEUKEMIA *IN VITRO*

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ABSTRACT

The survival rate of AML patients is remarkably low and this remains a challenge. Thus, finding novel treatments is urgently needed. Numerous studies have in-depth described how miRNA expression is frequently dysregulated in human acute myeloid leukemia (AML). This study aimed to evaluate the role of miR-155 gene silence on oncogenesis of acute myeloid leukemia cell lines via dysregulation of Src homology 2 domain containing inositol polyphosphate 5-phosphatase 1 (SHIP1) and CCAAT/enhancer binding protein beta (CEBPβ) genes expression to access the potential role of anti-miR-155 as a therapeutic target in acute myeloid leukemia. Total cell count and cell viability by using trypan blue staining assay showed a marked reduction of cell viability in miR-155 inhibitor transfected cells compared to MOCK cells. MTT assay showed that transfection of K562 cells with miR-155 inhibitor suppresses the cell proliferation and showed marked decline in tumor load. Moreover, cultured K562 cells showed marked reduction in the miR-155 gene expression level and marked increased in the CEBPB and SHIP1 genes expression levels compared to untreated cells. In conclusion the current study has validated the significance of knocked-down miR-155 expression in human AML cell line (K562). In addition, the current results suggested that miR-155 have an oncogenic role and inhibition of miR-155 was verified to have a tumor suppressive role. Thus miR-155 might be used as therapeutic target in acute myeloid leukemia patients in the future.

KEYWORDS: AML, MiRNAs, hematopoiesis, hematologic malignancies, SHIP1, CEBPβ.

1- INTRODUCTION

A common myelogenous cancer in adults, acute myeloid leukemia (AML) is frequently characterized by disease relapse. AML' s pathophysiological process is not yet fully understood.[1] Chemotherapy is the standard path of treatment for AML. Despite great advancement and success, a rising number of patients have developed a resistance to these treatments, mainly due to molecular mechanisms of drug resistance, necessitating the development of novel approaches to solve these problems.[2] Due to its potential to cure diseases at the molecular level, gene therapy has recently gained significant attention. Examples include gene silencing; mutation correction, and antiangiogenic and suicide gene therapy.[3]

A class of non-coding, regulatory RNAs known as microRNAs is responsible for regulating a number of vital physiological functions.[4] Dysregulated in cancer, miRNAs can either promote tumorigenesis or act as tumor suppressors. Studies have connected the pathophysiology of AML to miRNA alterations involved in gene regulation.[5]

In B cell lymphoma, the function of MicroRNA-155 (miR-155) as an oncogene is well-established; however, its function in AML is less clear. There is proof that miR-155 may either promote or suppress the development and spread of AML.[6] Overexpression of miR-155 in human hematopoietic stem cells improved proliferation of myeloid progenitors and inhibited differentiation into erythrocyes and megakaryocytes inducing polyclonal lymphocytosis in murine lymphocyte precursors, but it also caused high-grade lymphocytic leukemia.[7]

The differing amounts of miR-155 regulate a significantly diverse collection of gene targets, according to RNA-Sequencing study. MiR-155 directly binds to and inhibits Src homology-2 domain containing inositol 5-phosphatase 1 (SHIP1), a depressor of immune cell signaling.[6] ***Marcucci, et al.***'s.[8] investigation found that mice overexpressing miR-155 or lacking SHIP1 have

identical myeloid-proliferative illness symptoms, such as increased granulocyte-monocyte cells and decreased B-lymphocytes.[9]

According to a study on mice conducted by Liao and his colleagues, in addition to targeting SHIP1, miR-155 also targets CCAAT/enhancer binding protein beta (CEBPβ) which raises the possibility that aberrant miR-155 signaling may cause both SHIP1 and CEBPβ to be downregulated in AML.[10] In order to assess the potential of anti-miR-155 as an immunotherapeutic target in acute myeloid leukaemia, the study examined the effects of miR-155 gene silence on oncogenesis in myeloid leukemia cell lines via dysregulation of SHIP1 and CEBPβ.

2- SUBJECTS AND METHODS

An experimental *in vitro* study was conducted on K562 "human acute myeloid leukemia cells", in which the cells are transfected with miR-155 inhibitor. The cell line (K562) was purchased from Vacsera, Cairo, Egypt; the 3rd cell passage was used for the transfection experiment, followed by assessment of cytotoxic effect and miR-155 gene expression analysis. The efficacy of transfection was assessed by total cell counting, cell viability "Trypan blue" assay and Cell proliferation assay "MTT". Finally, miR_155, SHIP1 and CEBP genes expression were measured in harvested cell lines in transfected and untreated cells.

2.1. MiRNA transfection

K562 cells were subculture in 24-well on day before the experiment, a humidified atmosphere of 5% CO_2 at 37°C was used to seed 1–5 x 10^6 cells in Dulbecco's modified eagle medium (DMEM), which was supplemented with 10% fetal bovine serum (FBS), penicillin (100IU/mL), streptomycin (100µg/mL) and amphotericin-B (5µg/mL) (***Gibco, ThermoScientific, USA***). Before beginning any studies, cultures were examined under an inverted phase microscope. The next day, miRNA inhibitor complexes are added to wells followed by the addition of HiPerFect transfection reagent (***Qiagen, Germany***). The miR-155 inhibitor was utilized as a miRNA inhibitor at a dose of 50 nM. The cells are then given the complex of the miRNA- transfection reagent after it has been created. The transfection complexes were cultured with the cells under their usual growth conditions, and gene expression was observed 72 hours after transfection. By running a number of controls concurrently with the miR-155 inhibitor tests, the effectiveness of the transfection experiment was confirmed. Three controls were used: Mock cells (untreated K562 cells), miRNA inhibitor control (All Stars Negative Control siRNA; "mi-Script Inhibitor Negative Control" which targets the sequence of All siRNA, and has no homology to any known mammalian gene), and mi-Script inhibitor positive Control.

2.2. Viability staining (Trypan Blue) assay

Trypan Blue assay kit, available from **Thermo Fisher, USA** (cat no: 15250061), was used to identify viable (capable of growing) and nonviable cells in a culture. The "dye exclusion" principle underlies this staining technique, according to which live cells are those whose membranes exclude the dye (i.e., do not take it up). Trypsinization was used to extract the cells. The cells were resuspended in the proper volume of growth medium that had been pre-warmed, resulting in a cell density of at least 10^6 cells/ ml. 0.5 ml 0.4% (w/v) trypan blue were added and 0.3 ml PBS to 0.1 ml of the cell suspension. They were mixed thoroughly, and left standed for one to two minutes. alternatively, 0.4 ml of 0.4 % trypan blue were added directly to 0.4 ml of cells in growth medium. The stained and unstained cells were counted using a hemocytometer. Blue-stained cells are nonviable and unstained cells are viable.

No. of viable cells/ Total no. of cells = % viability

2.3. Assessment of cytotoxicity (MTT assay)

The cytotoxic effect on K562 cells followed the transfection with miR-155 inhibitor was assessed using the cell proliferation assay. The cell cytotoxicity assay was performed using ***the Vybrant® MTT Cell Proliferation Assay*** Kit, purchased from ***Thermo Fisher, Germany*** (cat no: M6494). The K562 cells (8×10^3 cells per well) were seeded in 96-well culture plates in and incubated at 37 °C with 5% CO_2 for 72 hours in DMEM media then 100µL of media were removed and replaced by new media. Twenty µL of MTT solution (1 mg/mL) (***Invitrogen, ThermoScientific, Germany***) were added to each well. And the plates were incubated at 37 °C and 5% CO_2 for four hours. Finally, the MTT solution was removed and 100 µL of SDS-HCL were added to the wells. Cell viability was determined by measuring the optical density at 570 nm on a spectrophotometer (***ELx800; Bio-Tek Instruments Inc., Winooski, VT, USA***).

2.4. Assessment of gene expression level of investigated parameters

2.4.1. RNA extraction and purification

Utilizing a miRNeasy Serum/Plasma Kit (cat. no. 217184) obtained from ***QIAGEN Germany***, total RNA was extracted from harvested cell lines. According to the manufacturer's directions and protocols, the extraction procedure was carried out. QIAzol Lysis Reagent was added to the harvested cell lines. Then chloroform was added and the cells were centrifuged**.** 100% ethanol was added to the upper aqueous phase layer so precipitation of RNA was occured. RNA pellet was washed by adding 500 µl of 80% ethanol to the RNA pellet. After that, the pellet was subjected to being dried by air. According to the pellet size, the dried pellet was dissolved in DEPC treated water. The quality and the concentration of RNA were checked using UV spectrophotometer by measuring the optical density of the samples at 260 nm and 280 nm. The purity of the RNA samples was determined from A_{260}: A_{280} ratio.

2.4.2. Reverse transcription of total RNA (cDNA synthesis)

The Total RNAs were reversibly transcribed using a miScript II RT kit purchased from ***Qiagen, Hilden, Germany*** (Cat. no. 218161). The reverse-transcription master mixture was prepared on ice for the first strand cDNA synthesis by mixing: 4 µl 5x miScript HiFlex buffer, 2 µl 10 x miScript dNTP mixtures, 2 µl miScript reverse transcriptase mixture and 7 µl RNase-free water. Exactly 5 µl of RNA sample was added and each tube was mixed gently by pipetting up and down. The tubes were briefly centrifuged (to spin down the contents and eliminate any air bubbles) and then loaded into the Thermal Cycler ***(Biometra, Germany).*** Reverse transcription and first strand cDNA synthesis were performed for one cycle (37 ºC for 60 min and 95 ºC for 5 min). The cDNA product of the reverse transcription was stored at -20 ºC until amplification.

2.4.3. Measurement of miR-155 gene expression in harvested cell lines

The miRNAs expression level was measured in harvested cells using Syber-green based Real-time PCR. MiR-155 expression level was measured using the miScript SYBR Green PCR Kit purchased from ***Qiagen, Germany*** (Cat. no. 218300) and miScript Primer Assay (Hs_miR-155_1; and Hs_RUN6; as a housekeeper gene ***(Qiagen, Germany)***. All samples were analyzed using the 5-plex Rotor-Gene Real-Time PCR Analyzer ***(Qiagen, Germany)***. The PCR reaction mix was prepared for a final volume of 18 µl per well as follows; 10 µl 2x QuantiTect SYBR Green PCR master mix, 2 µl 10x miScript universal reverse primer, 2 µl 10x miScript primer assay and 4 µl RNase-free water. Exactly 2 µl template cDNA were added and each well was mixed gently by pipetting up and down. The PCR plate was tightly sealed with the adhesive sheet. The real-time PCR cycler was adjusted as follows: initial denaturation at 95˚C for 15 min and 40 cycles (denaturation at 94˚C for 15 sec, annealing at 55˚C for 30 sec and extension at 70˚C for 30 sec). Melting curves were performed by rapid heating to 95˚C for 15 sec to denature the DNA, followed by cooling to 60˚C to assure the purity and specificity of amplified products.

2.4.4. Measurement of SHIP1 and CEBPB genes expression in harvested cell lines

The gene expression level was amplified from mRNA using Quantitect primer assay for each gene (Hs_SHIP1 and Hs_CEBPB primer assays) (***Qiagen, Hilden, Germany***), and the **ACTB_1_SG QuantiTect Primer Assay** (β-actin) (cat no: 249900), as housekeeper gene. All samples were analyzed using the 5 plex Rotor Gene PCR Analyzer (***Qiagen, Germany***). The PCR reaction mix was prepared by adding as follow, 2x QuantiTect SYBR Green PCR Master Mix, 10x QuantiTect Primer Assay, template cDNA, and RNase-free water were thawed at room temperature (15–25˚C). Then, the reaction mix was prepared for a final volume 18 µl per well reaction volume as following: 10 µl of 2x QuantiTect SYBR Green PCR Master Mix, 2 µl 10x miScript universal primer, 2 µl 10x Quantitect Primer Assay and 4 µl RNase-free water. The reaction mix was mixed thoroughly but gently, and dispensed appropriate volumes into the Rotor-Disc wells then 2 µl template cDNA were added, to reach 20 µl as final volume. Carefully, tightly the disc was sealed with Rotor-Disc Heat-Sealing Film.

Consequently, the real-time cycler initial was programmed as: activation step for 15 minutes at 95˚C for Hot-Star Taq DNA Polymerase activation. Three-step cycling: denaturation for 15 seconds at 94ºC, annealing for 30 seconds at 55ºC, extension for 30 seconds at 70ºC, for 40 cycles. Moreover, the expression levels were normalized to β-actin levels as a reference gene. The relative gene expression levels (fold change) were normalized to an internal control (β-actin) and relative to calibrator (negative control sample) were calculated using the equation $\mathbf{2^{-\Delta\Delta Ct}}$ test/ control.

2.5. Statistical analysis

The collected data was revised, coded, tabulated and introduced to a PC using Statistical Package for Social Science **(SPSS 23 for Windows; SPSS Inc, Chicago).** Data were presented and suitable analysis was done according to the type of data obtained for each parameter. The data are presented in Mean, and range for parametric numerical data, in frequency and percentage for non-numerical data. The independent- **t**-Test was used to assess the statistical significance of the difference between two study group means. The statistical significance is set at value were $p<0.05$.

3. RESULTS

3.1. Comparison between miR-155 transfected K562 cell and un-treated cells for the total cell count and cell viability

Cell count was applied for the transfected cells using the hemocytometer, our results revealed that untreated K562 cells showed a high cell count (figure.2.A) compared to miR155 inhibitor transfected cells (figure.2.B), the mean cell count of MOCK and miR-155 inhibitor were $6.85x10^5$ and $4.93x10^4$, respectively. Non-viable cells in miR-155 inhibitor and MOCK were $1.94x10^2$ and $1.90x10^2$ respectively see (figure 1). The percentage of viable cells in treated cell line was calculated based on MOCK (untreated cells). Our results showed a marked reduction of cell viability in miR-155 inhibitor transfected cells when compared to MOCK cells. Cell viability was assessed using Trypan blue stain see (figure 2.C&D).

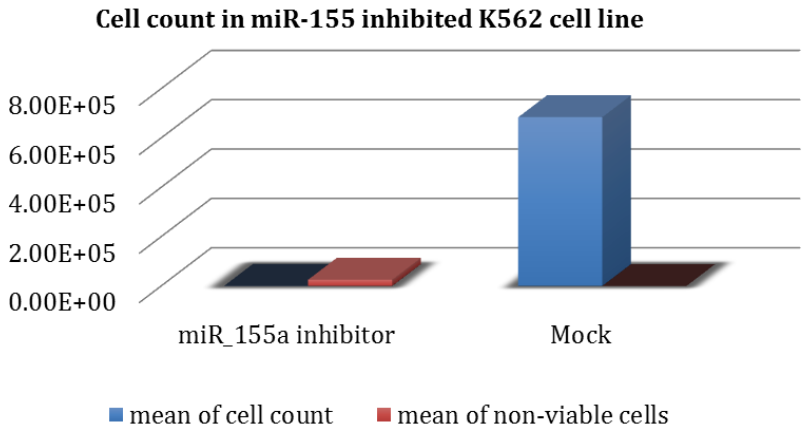

Figure 1: Comparison between the mean of cell count and non-viable cells in K562 cells transfected with miR-155 inhibitor.

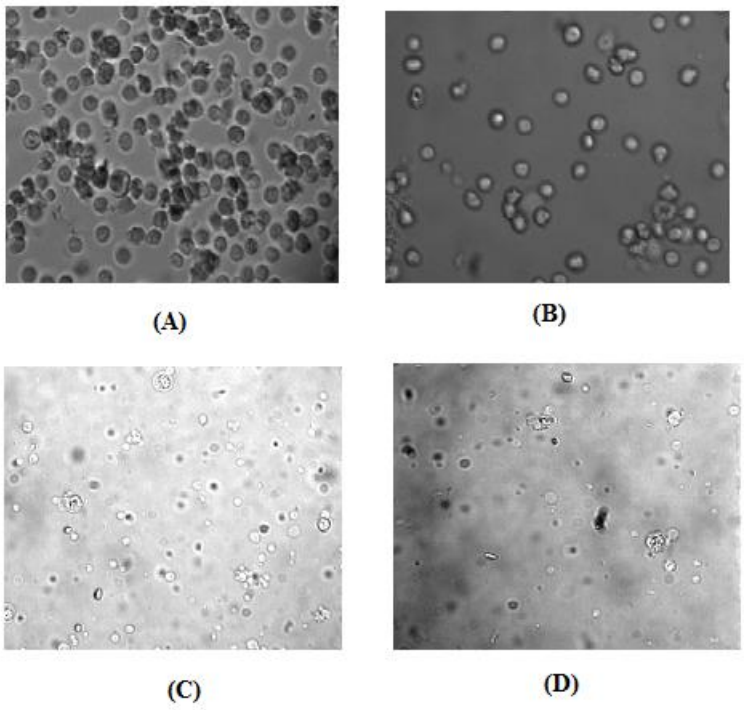

Figure 2: (A) K562 cells cultured in DMEM at 37°C and 5% CO_2 for 48 hours; image was captured by inverted microscope (Labomed, USA). (B) miR-155 knocked K562 cells cultured in DMEM at 37°C and 5% CO_2 for 48 hours; image was captured by inverted microscope (Labomed, USA). (C) Cell count by Trypan blue stain: Untreated K562 cells and cultured in DMEM at 37°C and 5% CO_2 for 48 hours, image was captured by inverted microscope (Labomed, USA). (D) Cell count by Trypan blue stain: K562 cells transfected with miR-155 inhibitor and cultured in DMEM at 37°C and 5% CO_2 for 48 hours, image was captured by inverted microscope (Labomed, USA).

3.2. Comparative analysis for percentage of inhibition between miR-155 transfected- K562 cells and untreated cells

To investigate the impact of transfection with miR-155 inhibitor, an MTT assay was conducted on treated and untreated cells to assess the effect of inhibition on cell proliferation and growth. The percent of cell proliferation inhibition was calculated based on the proliferation of MOCK cells (untreated cells).

Our results showed that transfection of K562 cells with miR-155 inhibitor suppresses the cell proliferation and showed marked decline in tumor load, MTT assay demonstrated that the percentage of inhibition for miR-155 inhibited cells was 85% compared to untreated cells **(**Figure 3).

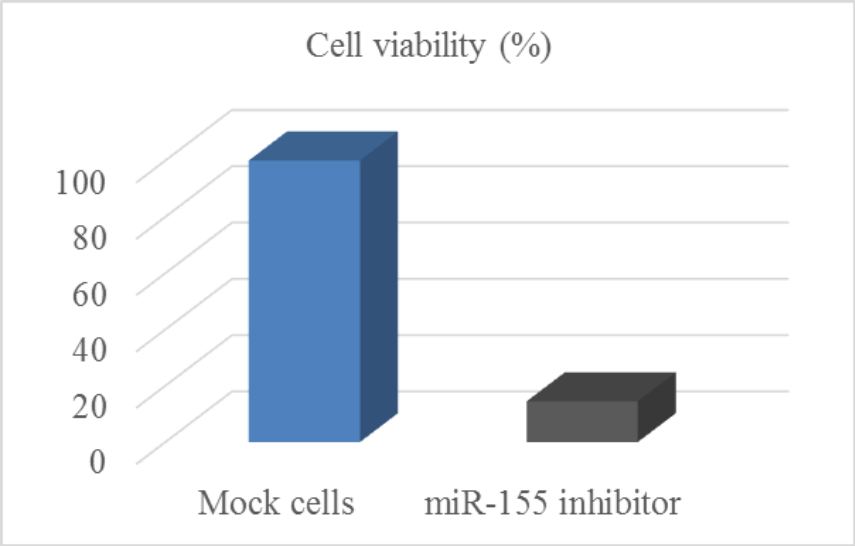

Figure 3: The percentage of cell viability in K562 cells transfected with miR-155 inhibitor compared to untreated Mock cells.

3.3. Comparative analysis for the fold change of miR-155 expression level between miR155 transfected K562 cells and untreated cells

MiR-155 expression was measured in cultured cell lines after transfection with miR-155 inhibitor using qPCR; the expression level was normalized to MOCK cells. Our results revealed that all cultured K562 cells shows marked reduction in the miR-155 gene expression (figure 4).

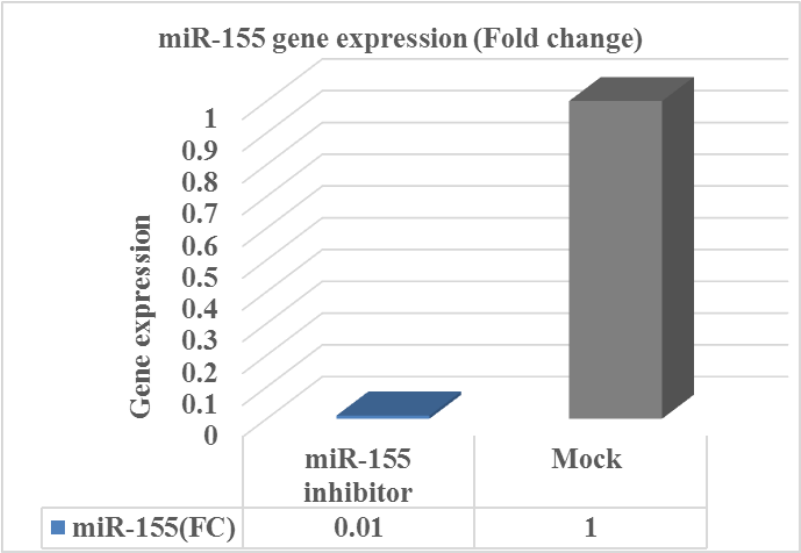

Figure 4: MiR-155 gene expression (Fold change) in miR-155 knocked down K562 cells compared to untreated Mock cells.

3.4. Comparative analysis for the expression of CCAAT/enhancer binding protein beta (CEBP) and SHIP1 genes expression (Fold change) in K562 cells transfected with miR-155 inhibitor & untreated cells

The CEBP and SHIP1 mRNA genes expression were measured in cultured cell lines after transfection with miR-155 inhibitor using qPCR; the expression level was normalized to MOCK cells. Our results revealed that cells transfected with miR-155 inhibitor showed marked increased in the CEBP expression by 4.4 folds compared to the untreated cells. On the other hand, the SHIP1 mRNA gene expression was markedly increased by 16.7 folds compared to the untreated cells (Figure 5).

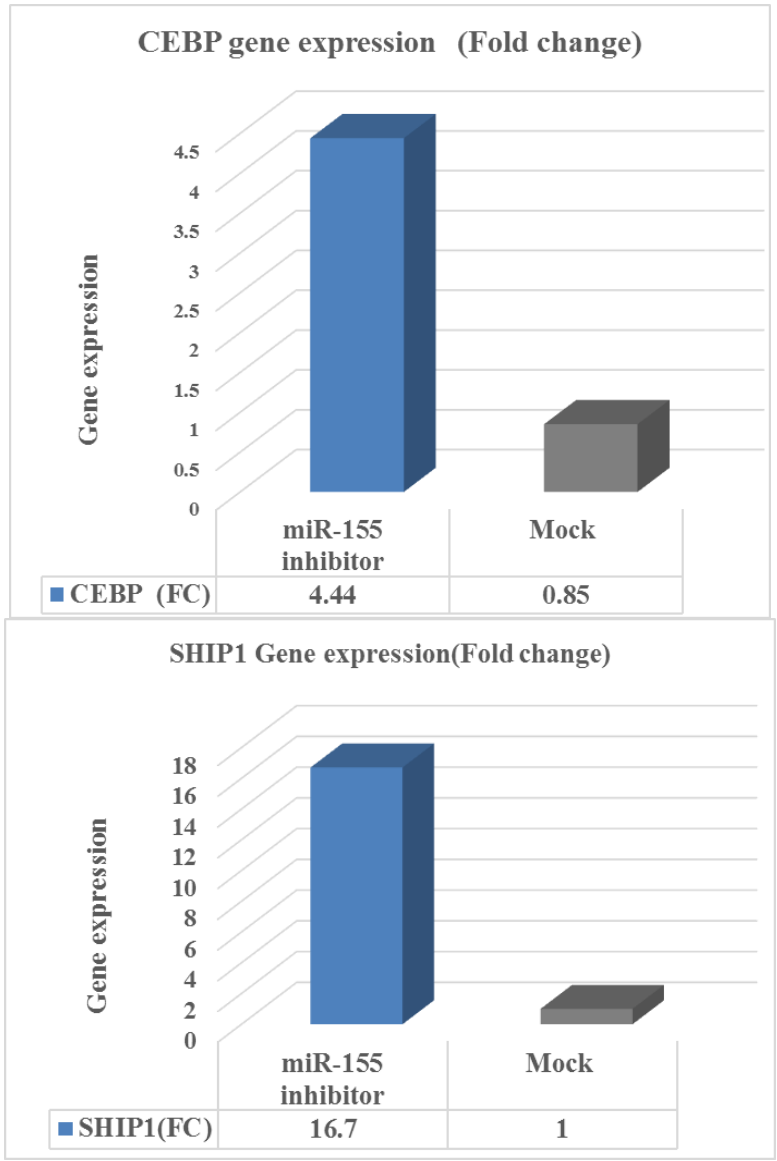

Figure 5: CEBP and SHIP1 genes expression (Fold change) in K562 cells transfected with miR-155 inhibitor, compared to untreated Mock cells.

4- DISCUSSION

The B-cell integration cluster (*BIC*) gene, which is located on chromosome 21, produces a noncoding RNA that is used to process MiR-155. It is referred to as miR-155-5p (from the 5′ arm) or miR-155-3p (from the 3′ arm) depending on which arm of the pre-miRNA-155 hairpin structure it is formed from. Multiple biological processes have been linked to the multifunctional miRNA molecule MiR-155. About 140 genes are on the list of miR-155 targets, including genes that code for inflammatory proteins, tumor-suppressor genes, and regulatory proteins for myelopoiesis and erythropoiesis.[11]

One of the most frequently overexpressed miRNAs in both hematological and solid tumors is miR-155. It has been discovered that aberrant expression of miR-155 is connected to a number of hematological cancers. Depending on the type of malignancy and the characteristics of the tissue, it can either have an oncogenic or a tumor-repressor impact.[12]

Investigations on the effects of miRNA-155 knockdown in children with acute lymphoblastic leukemia (ALL) in HCV patients have revealed that miR-155 is a crucial miRNA and is a target for treatment in patients with HCV childhood leukemia.[13]

The oncogenic miR-155 which is translated from the noncoding RNA BIC (B-cell Integration Cluster) is one of the miRNAs in B-cell malignancies that has been the focus of the most research. Since the biogenesis of miR-155 has already been extensively in-depth reviewed by others, just a quick summary is provided. MiR-155 plays a critical role in hematopoiesis, the immunological response, and inflammation under normal physiological settings.[14]

For the miR-155-mediated pathogenesis of hematological malignancies, various pathways have been proposed. One of these postulated pathogenic processes involves miR-155' s downregulation of B-cell lymphoma 6 protein (BCL6) and histone deacetylase 4 (HDAC4) which is particularly relevant for lymphomas like diffuse large B-cell lymphoma (DLBCL).[15] Another study postulated that BCL6 and HDAC4 expression are adversely linked with miR-155 expression.[16]

It has been discovered that it is elevated in a number of cancer types and has demonstrated a particular role in the pathogenesis of B-cell malignancies. The target genes of miR-155 and the associated underlying molecular pathways are responsible for its carcinogenic effect. Mice with miR-155 overexpression develop lymphoproliferative illnesses, however when it is removed, the disorders remit. So, miR-155 is recommended as a potential therapy target in the future. Numerous researches have looked into its potential as a biomarker in a variety of B-cell cancers, but the results have been conflicting.[17]

In light of the aforementioned fact, it was intended for the current investigation to examine the impact of miR-155 suppression on cell proliferation in human acute myeloid leukemia cells (K562) in order to assess the potential therapeutic benefits of anti-miR-155 in AML patients. As a result, a miR-155 inhibitor was transfected into an acute myeloid leukemia cell line, and the cellular load in leukemic cell lines was measured in the treated cell lines and compared to untransfected cell lines. The effectiveness of miR-155 knock-down on proliferation rate was also tested using a mitogenic cell proliferation assay. Finally, miR-155 expression levels were measured in miR-155 knocked down cells and untreated cells using specific miR155 specific primer sequence and quantitative real time PCR (qPCR).

Furthermore, miR-155 overexpression inhibited cell proliferation, caused cell cycle arrest, and induced cell apoptosis, whereas miR-155 inhibitors facilitated cell proliferation, cell cycle progression, and decreased apoptosis. According to additional research, HT-29 cell's ability to migrate and invade was decreased by miR-155, but this ability was enhanced by miR-155 inhibitor. These findings provided evidence for miR-155 and collagen triple helix repeat containing 1 (CTHRC1) as a novel anti-onco molecular target for the treatment of colorectal cancer in the future by preventing colorectal cancer progression and metastasis via silencing CTHRC1*in vitro*.[18]

Children with acute lymphoblastic leukemia (ALL) were shown to have very high levels of miR-155a, which was associated with a high risk of relapse and poor response to therapy. This suggests that miR-155 is involved in the pathogenesis of ALL and high-grade lymphoma. MiRNAs regulate expression of mRNA post transcriptionally and affect the direction of lymphoid precursor maturation in particular stages of lymphopoiesis. One previous study using transgenic mice discovered that over-expression of miR-155 causes a lympho-proliferative disease resembling the human diseases.[19]

Any aberrant miRNA expression can lead to leukemic malignancies and serve as a marker for various subtypes of ALL. MiR-155 expression was linked to lower overall survival in a recent meta-analysis of leukemia patients, and its potential as a predictive biomarker for this patient population was recognized.[20]

MiR-155a was discovered to be significantly expressed in patients with high-risk ALL.[21] An earlier study also established that downregulation of miR-155 expression was linked to a poor prognosis in patients with cytogenetically abnormal acute myeloid leukemia (AML). Which was consistent with our results that miR-155 inhibitor acts as a tumor suppressor in the pathogenesis of AML and was significantly correlated to the survival of patients with AML.

Studies discovered a feedback loop between p53 and miRNA-155a that contributes to chemotherapy resistance and which can be targeted to improve outcomes for lung cancer patients. It was also discovered that combining anti-miR-155a with chemotherapeutics like doxorubicin improved the sensitivity of malignant cells to chemotherapy, which sharply reduced their rate of proliferating.[22]

The possibility of miR-155 as a therapeutic target in B-cell malignancies has been discussed in another study. Given that the results were consistent across numerous investigations, MiR155 expression may be an important tool for the diagnosis and prognostic evaluation of DLBCL patients as well as for chronic lymphocytic leukemia (CLL).[23]

Elevated miR-155 expression was generally linked to more severe disease and poor prognosis, but this association was not statistically significant across all investigations. In the final analysis, stratifying AML patients based on specific prognostic variables was not consistently possible using miR-155 expression. As a direct predictive biomarker for AML, higher miR-155 expression as an independent factor was related with poor clinical outcomes, suggesting its potential to serve as a direct prognostic biomarker in AML. Considering the fact that miR-155 is an oncomir.

It has been shown that miR-155 binds to 3` UTR of SHIP1 and CEBPB mRNAs, which prevents SHIP1 and CEBPB from being translated. Studies have found a strong correlation between myeloproliferative disorders (MPDs) caused by miR-155 expression and targeted suppression of CEBPB and SHIP1. Mice studies revealed that miR-155 targets SHIP1 and CEBPB. Therefore, the development of human MPDs and

myeloid leukemia, in which miR-155 has been found to be overexpressed, may be influenced by the reduction of SHIP1 and CEBPB expressions by miR-155.[24] Additionally, hematopoietic cells overexpressing miR-155 in *vitro* and in *vivo* have lower levels of endogenous SHIP1and CEBPB expression. This results in higher AKT activation and decreased apoptosis, which promotes the development of leukemia.[25] These outcomes are consistent with our current findings. The expression of SHIP1 and CEBPB was significantly enhanced in cells transfected with miR-155 inhibitor relative to untreated MOCK cells, as was the case when miR-155 was reduced with miR-155 inhibitor in K562 human cell lines.

CONCLUSION

In conclusion, the current study has been validated the significance of knock-down miR-155 expression in human AML cell lines (K562). In addition, it was also suggested that miR-155 have an oncogenic role and inhibition of miR-155 was verified to have a tumor suppressive role. Taken together, these findings implicated that miR-155 might be used as therapeutic target in acute myeloid leukemia patients.

Abbreviations

AML: acute myeloid leukemia; SHIP1: Src homology 2 domain containing inositol polyphosphate 5-phosphatase 1; CEBPβ: CCAAT/enhancer binding protein beta; DMEM: dulbecco's modified eagle medium; FBS: fetal bovine serum; *BIC*: B-cell integration cluster; ALL: acute lymphoblastic leukemia; BCL6: B-cell lymphoma 6 protein; HDAC4: histone deacetylase 4; DLBCL: diffuse large B-cell lymphoma; CLL: chronic lymphocytic leukemia; MPDs: myeloproliferative disorders; CTHRC1: collagen triple helix repeat containing 1.

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