Original Article

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Investigating Apoptotic Effect through Blocking miR-181b and miR-222 Using LNA-anti-miRNA in HL-60 Cell Line: Strategies to Improve Hematopoietic Stem Cell Transplantation

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ABSTRACT

Background: Several genes that control the commitment and differentiation of hematopoietic stem cells are regulated by miRNAs. Hematologic cancers like acute myeloid leukemia (AML) have been found to express miRNAs abnormally.

Objective: In this current study, we assessed the apoptotic effect of miR-181b and miR-222 blockage, which can influence the expression of *WT1*, *CEBPA*, and *C-KIT* genes in an HL-60 cell line.

Methods: Relative gene expression was observed by the SYBR Green Real-Time PCR method. By transfecting the HL-60 cell line with locked nucleic acid (LNA)-anti-miRNA, miRNA expression was suppressed. MTT assay was used to determine the viability of transfected cells, and PE Annexin V apoptosis detection kit I was used to evaluate the apoptosis.

Results: After LNA transfection, the results showed a reduction in the expression of miR-181b and miR-222. The flow cytometry data showed the apoptosis reduction by the inhibition of miR-181b and apoptosis increase by the inhibition of miR-222. We also found that miR-222 inhibition dramatically reduced c-KIT level, however, miR-181b blockage was associated with up-regulated of *C-KIT* expression. Moreover, the LNA-modified miR-222 could up-regulate *BAX* and down-regulate *Bcl-2*, whereas, after the transfection of the LNA-anti-miR-181b, *BAX* expression levels were significantly lower on average.

Conclusion: We concluded that the inhibiting of miR-222 and increasing miR-181b could help to control AML disease. MiR-222 could be a possible prognostic biomarker in patients who had hematopoietic stem-cell transplantation (HSCT) due to its higher expression in HSCT patients who got a graft-versus-host disease (GVHD).

INTRODUCTION

Acute myeloid leukemia (AML) is a type of leukemia that causes bone marrow failure and immature myeloid cell growth [1]. It is one of the most prevalent hematologic cancers in adults [2] and is brought on by myeloid stem cells that proliferate and differentiate abnormally [3]. It results from two or more genetic changes, and these alterations activate signal transduction pathways, which promote proliferation and act on transcription factors while blocking differentiation [4].

Patients with hematologic malignancies, particularly AML, have been found to have dysregulated expression of miRNAs due to mutations or epigenetic alterations [5].

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Hematopoietic stem cell transplantation (HSCT) is a prominent curative therapy that enables the replacement of the host's hematopoietic stem cells (HSCs) with those from a healthy donor or genetically modified autologous HSCs. However, graft-versus-host disease (GVHD), which affects recipients of allogeneic HSCT, remains the leading cause of post-transplant morbidity and mortality [6]. Recent studies have highlighted the potential of various microRNAs (miRNAs) as biomarkers for predicting GVHD following allogeneic hematopoietic cell transplantation (HCT). Specifically, a plasma profile comprising four miRNAs—miR-423, miR-199, miR-93*, and miR-377—has been identified as a predictor of acute GVHD (aGVHD). Furthermore, this miRNA signature has been associated with both the severity of aGVHD and patient survival outcomes [7, 8].

Small, non-coding RNA molecules known as microRNAs (miRNAs) are recognized as crucial regulators of practically all physiological signaling pathways [9]. These molecules typically attach to the 3′ untranslated regions (3′ UTR) of their respective targets to impede translation or cause the instability and destruction of their mRNA targets [10]. MiRNAs have essential roles in every facet of cancer biologies such as proliferation, differentiation, and apoptosis [11]. Abnormal miRNA expression has also been observed in hematologic malignancies [12-14]. They play effective roles in all phases of hematopoiesis, such as the maintaining of stem cells, differentiation, proliferation, and apoptosis [15]. MiRNAs may act as tumor suppressors or oncogenes in leukemia [16, 17]. MiRNAs have been discovered to have a prognostic value for chemotherapy response and prognosis in cancer patients [18].

Previous research revealed that not all AML patients exhibit the same micro-RNA expression. Some of them, like miR-155 [19] and miR-196b, are overexpressed [20, 21]. Others, such as miR-29a, -29b, and -29c, however, are downregulated [22]. In AML patients, several microRNAs can be employed as biomarkers for diagnosis or prognosis. For example, miRNA-181a-3p, miR-125a and -125b, miR-93, and miR-98 overexpression are associated with favorable prognoses in AML patients [23-27]. However, the upregulation of some additional genes, such as miR-486 [28], miR-362-5p, and miR-21, can also be associated with a bad prognosis [29, 30]. However, the downregulation of some additional genes, such as miR-133 [31], miR-29a, -29b, and -29c are linked to a bad prognosis [32]. In this study, we focused on miR-181b and miR-222 which are among well-researched miRNAs in AML that are undergoing significant changes [33, 34]. Several studies have demonstrated that AML patients have dysregulated miR-222 and miR-181b expression [35, 36]. There is growing evidence that the miR-181 family regulates the development of hematopoietic cells such as B cells, T cells, natural killer (NK) cells, and megakaryocytes [12, 37]. Previous research suggested that the adaptor miR-181b may be essential in the connection between inflammation and malignant transformation [38]. Additionally, it found that miR-181b might control the proliferation of myeloid cells, which is crucial for the development of the AML condition [39]. Increased levels of miR-181b prevented cancer cells from growing, spreading, migrating, and spreading to other parts of the body. This was observed in a variety of malignancies such as chronic lymphocytic leukemia, cervical cancer, ovarian cancer, and gastric adenocarcinomas [40, 41]. MiR-222, however, has significant roles in tumorigenesis. Studies showed the enhancement of cancer cell biological mechanism, migration, and microtubule formation by miR-222, which can lead to promoting tumor cell proliferation. In the future, miR-222 may be used as a therapeutic target for AML patients [42, 43].

It have shown that microRNAs (miRNAs) target a diverse range of messenger RNAs (mRNAs) and can function as tumor suppressors, oncomiRs, or regulators of apoptosis across various types of tumors. Specifically, certain miRNAs are associated with promoting apoptosis, while oncomiRs facilitate the progression of maliganancies [44]. OncomiRs and apoptosis can be used as tumor biomarkers for diagnosis, prognosis, and choosing the

best treatment plan for cancer patients by better understanding their functions in certain forms of cancer [45]. Based on the evidence, overexpression of miR-181b and miR-222 has been associated with an increase and decrease in apoptosis, respectively [41, 43]. However, more studies need to clear the probable regulatory effect of miR-181b and miR-222 on apoptosis in AML patients.

In this current study, we assessed the apoptotic effect of miR-181b and miR-222 in the HL-60 cell line. We evaluated the apoptotic index changes and cell viability in the HL-60 cell line after miR-181b and miR-222 knockdown by locked nucleic acid (LNA). LNA is a specific type of modified RNA nucleotide with probable therapeutic applications that recently have been employed to suppress miRNAs [46].

Moreover, we will discuss the effect of miR-181b and miR-222 blocking on the regulation of the essential molecules involved in the intrinsic mitochondrial apoptosis pathway such as *BAX*, *BCL-2*, and *MCL-1* genes. The Bcl-2 family includes both pro-apoptotic proteins like Bcl-2 associated X protein (Bax), and anti-apoptotic proteins such as Bcl-2, and myeloid cell leukemia 1 (Mcl-1). Together, these are central in regulating the intrinsic apoptotic pathway [47].

There are five classes of genes' mutations associated with myeloid malignancy: Class I; signaling pathways-related genes (FMS-related tyrosine kinase 3 [*FLT3*], *C-KIT*, and casitas b-lineage lymphoma [*CBL*]), class II; transcription factors-related genes (CCAAT/enhancer-binding protein alpha [*CEBPA*] and nucleophosmin1 [*NPM1*]), class III; epigenetic modification-related genes (enhancer of zest homolog 2 [*EZH2*], DNA-methyltransferase 3 alpha [*DNMT3A*], and isocitrate dehydrogenase 1/2 [*IDH1/2*]), class IV; tumor suppressor genes (Wilms' tumor 1 [*WT1*]) [48].

In this study, we investigated the effect of blocking miR-222 and miR-181b on the expression of *WT1*, *CEBPA*, and *C-KIT* in the HL-60 cell line.

We aimed to understand the regulatory effects of miRNAs which may describe a novel signaling pathway of promotion cancer cell apoptosis and quiescence.

MATERIALS AND METHODS

Cell Culture

The National Cell Bank of Iran provided the HL-60 cell line (Human Acute Promyelocytic Leukemia: APL) (Pasteur Institute, Tehran, Iran). The cells were kept in 25 cm^2 culture flasks (Nunc, Denmark) at 37°C in Roswell Park Memorial Institute (RPMI) 1640 (Gibco, UK) supplemented with 10–20 percent fetal bovine serum (FBS; Gibco), 100 U/mL of penicillin, and 100 g/mL of streptomycin (Sigma-Aldrich, USA). To keep the cells in an exponential growth phase, the cells underwent passage twice a week.

SYBR Green Real-time PCR

Relative expression of the miR-181b and miR-222 mRNAs was assessed by using an iQ5 thermocycler (BioRad Laboratories, USA) and SYBR® Premix Ex Taq TM II (Tli RNaseH Plus) master mix (Takara, Japan), and specific primers for each miRNA. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the internal control gene to standardize the expression of the miR-181b and miR-222 mRNAs. The Real-Time PCR reaction methodology and primer sequences are summarized in Table 1. After the program, a melt curve analysis was done to establish the reaction's specificity. The [$2^{-\Delta\Delta Ct}$] method, where Ct= [Ct (patient) - Ct (control)] and Ct= [Ct (sample) - Ct (housekeeping gene)], was used to calculate the changes in the relative expression levels of MiR-181b and MiR-222 mRNAs. At the very least, duplicate wells were used for every real-time polymerase chain reaction.

Cell Transfection

The LNA was used to block the expression of miR-222 and miR-181b since it is precisely complementary to their 5′ regions. The miRNAs' nucleotide sequences, 5′-CUCAGUAGCCAGUGUAGAUCCU for miR-222 and 5′-AACAUUCAUUGCUGUCGGUGGGU

Table 1: Sequences of primers and thermocycling condition

Primers	Sequences (5′-3′)	Thermocycling condition
miR-222	Forward: 5′-GCATGTCATCACTCAGTAGCCAGTGTA-3′ Reverse: 5′- CCAGTGCAGGGTCCGAGGTA-3′	94°C/2 min, 40 cycles of 95°C/30 sec, 57.5°C/ 20 sec and 70°C/30 sec
miR-181b	Forward: 5′- GTTTGAACATTCATTGCTGTCG-3′ Reverse: 5′- GTGCAGGGTCCGAGGT-3′	94°C/2 min, 40 cycles of 95°C/30 sec, 58°C/20 sec and 70°C/30 sec
WT-1	Forward: 5′- CCAGGCTTTGCTGCTGAG-3′ Reverse: 5′- GTGGCTCCTAAGTTCATCTG-3′	95°C/2 min, 40 cycles of 95°C/30 sec, 57.5°C/ 20 sec and 70°C/30 sec
C-KIT	Forward: 5′- TTCTGCTCCTACTGCTTC-3′ Reverse: 5′- CTGGATGGATGGATGGTG-3′	95°C/2 min, 40 cycles of 95°C/30 sec, 59.5°C/ 20 sec and 70°C/30 sec
CEBPA	Forward: 5′- GAAGCACGATCAGTCCAT-3′ Reverse: 5′- GCCAGATACAAGTGTTGATAT-3′	95°C/2 min, 40 cycles of 95°C/20 sec, 59.5°C/ 20 sec and 70°C/30 sec
GAPDH	Forward: 5′- GGACTCATGACCACAGTCCA-3′ Reverse: 5′- CCAGTAGAGGCAGGGATGAT-3′	95°C/2 min, 40 cycles of 95°C/30 sec, 58.5°C/ 20 sec and 70°C/30 sec
BAX	Forward: 5′- GCCCTTTTGCTTCAGGGTTTCA-3′ Reverse: 5′- CAGCTTCTTGGTGGACGCAT-3′	94°C/2 min, 40 cycles of 94°C/30 sec, 60°C/20 sec and 72°C/30 sec
BCL-2	Forward: 5′- ACGAGTGGGATGCGGGAGATGTG-3′ Reverse: 5′- GCGGTAGCGGCGGGAGAAGTC-3′	94°C/2 min, 40 cycles of 94°C/30 sec, 60°C/20 sec and 72°C/30 sec
MCL-1	Forward: 5′-CCAGGCAAGTCATAGAAT-3′ Reverse: 5′-GAGGCTTACAGTCATAGTT-3′	65°C/2 min, 40 cycles of 94°C/30 sec, 56.5°C/ 30 sec and 72°C/30 sec

for miR-181b, were downloaded from www.mirbase.org. Life Technologies provided the LNA-miRNA inhibitors for miR-222, miR-181b, and the scrambled negative control oligonucleotides for microRNA inhibitors (Applied Biosystems, UK). For cell transfection, HL-60 cells (2.5×10^5 cells) were grown in a 6-well plate and attained 80% confluence after 24 hours. Following that, cells were transfected with 50 pmol of LNA-anti-miRNA using Invitrogen's lipofectamine 2000 reagent in serum-free RPMI 1640 medium following the manufacturer's instructions. Following transfection, the medium was changed to fresh complete medium (RPMI with 10% FBS, 100 U/ml streptomycins, and 100 ug/ml penicillin) at 37 °C in a humid environment with 5% CO_2 (approximately 7 hours). For real-time quantitative PCR analysis and cell viability measurement, the plates were placed in the refrigerator for up to 48 and 72 hours, respectively

Reverse Transcriptase microRNA Real-time PCR

The total RNA of the HL-60 cell line transfected with LNA-anti-miRs was extracted 48 hours after transfection using TRIZOL reagent and then converted into cDNA using Prime Script RT Reagent Kit to check the blocking level of MiR-181b and MiR-222 by LNA-anti-miRs (Takara, Japan). Then, Real-time PCR was carried out in an iQ5 thermocycler (BioRad Laboratories, USA) with specific miR-222 and miR-181b designed primers using SYBR® Premix Ex Taq TM II (Tli RNaseH Plus) master mix (Takara, Japan).

The results were calculated by using the Ct method as previously described [49].

Measurement of Cell Viability

The vitality of the cells was evaluated using the MTT test after 72 hours of transfection (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) (Sigma, Germany). In 96-well plates, a total of 5×10^3 cells were seeded and cultured for 24 hours at 37°C with 5% CO_2. Then MTT was added, and the incubation process was continued for an additional 4 hours at 37°C. Following the formation of formazan crystals, 200 µL of dimethyl sulfoxide (DMSO) was added, and the cells were then incubated for an additional 30 minutes at 37°C with moderate shaking. Finally, a microplate reader (FLUOstar Omega, BMG LABTECH, Germany) was used to assess the absorbance intensity at 570 nm. The absorbance ratio between the test groups and the control group was used to determine cell viability [50].

Expression of the BAX, MCL-1, and BCL-2

Total RNA from a transfected HL-60 cell line with LNA-anti-miRNA and scrambled LNA, as well as non-transfected cells, were extracted and transformed into cDNA using Prime Script RT Reagent Kit following effective miR-181b and miR-222 blocking and confirmation of cell transfection (Takara, Japan). Then, Real-time PCR was carried out in an iQ5 thermocycler using SYBR® Premix Ex Taq TM II (Tli RNaseH Plus) master mix (Takara, Japan) and particular *BAX*, *MCL-1*, and *BCL-2* designed primers (BioRad Laboratories, USA). The internal control was the GAPDH gene. Beacon Designer software and Primer 3 online software were used to create specific primers. The [$2^{-\Delta\Delta Ct}$] method was used to calculate the changes in the relative expression levels of *BAX*, *MCL-1*, and *BCL-2* mRNAs.

Apoptosis Analysis by Flow Cytometry

In the 6-well plates, 2.5×10^5 HL-60 cells were seeded. After 24 hours, transfection was done. Cells were collected 48 hours later using a dissociation buffer and then subjected to an apoptosis evaluation procedure using PE Annexin V apoptosis detection kit I (BD Inc. USA), per the manufacturer's instructions. FlowJow analysis software was used for the analysis. The proportion of Annexin V^+/7-AAD^- and Annexin V^+/7-AAD^+ stained cells in the population was used to calculate the levels of early and late apoptosis. The percentage of overall apoptosis was calculated as the sum of the early and late apoptosis rates.

Ethical Considerations

All stages were approved by the Ethics Committee of Shiraz University of Medical Sciences (Code: IR.SUMS.REC.1396.S42). 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.

Statistical Analysis

Version 18 of SPSS software was used to analyze the data. Using a student t-test, the mean expression levels of MiR-181b and MiR-222 were compared between patients and controls. The Pearson correlation test analyzed the correlation between MiR-181b and MiR-222 expression and laboratory data. The expression level of MiR-181b and MiR-222 was compared between patients according to response to chemotherapy treatment, cytogenetic aberration, and FAB subtypes by independent t-test. By using the 2-Related-Samples Test, the expression levels of MiR-181b and MiR-222, *BAX*, *MCL-1*, and *BCL-2* were compared between two groups (before and after transfection). Statistics were considered significant for P-values under 0.05.

RESULTS

LNA-anti-miR-181b and LNA-anti-miR-222 Effectively Inhibit miR-181b and miR-222 Expression

The mean expression levels of miR181b and miR-222 were compared between the LNA-anti-miR transfected, scrambled LNA (as a negative control), and non-transfected groups 48 hours after cell transfection to assess the effectiveness of miRNA blocking by

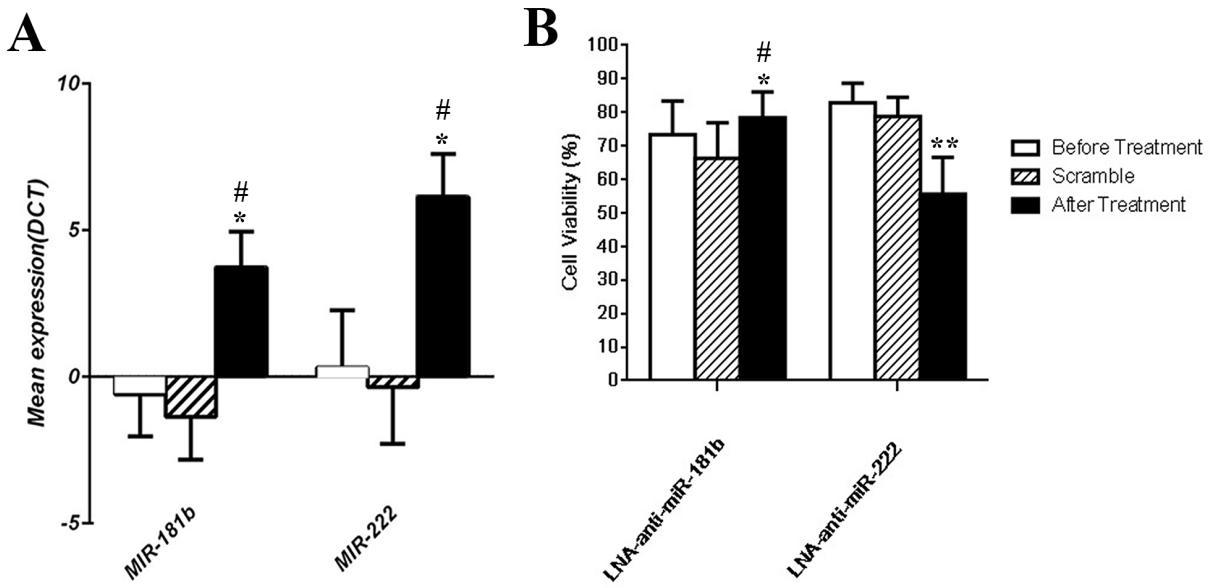

Figure 1: Changes in miR-181b and miR-222 expression after LNA-anti-miRNA transfection of HL-60 cells (**A**), and the impact of LNA-anti-miR-mediated blockade of miR-181b and miR-222 on cell viability (**B**).
*P< 0.05, **P≤ 0.01 vs non-transfected; #P< 0.05, ##P≤ 0.01 vs scrambled LNA.

LNA-anti-miRNA. Both miRNA levels decreased a bit in scrambled LNA-transfected cells compared to untreated cells. When compared to either the scrambled LNA group or the untreated group, the expression of miR-181b significantly decreased (3.71.2 vs. -0.611.4, respectively; P=0.03) (Fig. 1A). MiR-222 levels were statistically significantly lower following LNA transfection compared to the untreated group (6.11.4 vs. 0.311.9, respectively; P=0.02, Fig. 1A).

Viability of HL-60 Cell Line after LNA-anti-miR-181b and 222 Transfection

The HL-60 cell line's viability was reduced slightly in the scrambled LNA groups compared to the untreated control groups. However, a remarkable decrease was observed in the LNA-anti-miR-222 transfected group (about 55%) compared to control groups after 72 hours of transfection (P=0.01, Fig. 1B). Conversely, the blockage of the miR-181b significantly increased cell viability (P=0.04, Fig. 1B).

BAX, BCL-2 and MCL-1 Expression after LNA-anti-miR-181b Transfection

After HL-60 cell lines were successfully transfected with LNA-anti-miRNA to suppress miR-181b, the expression of the *BAX*, *BCL-2*, and *MCL-1* genes was assessed. According to the results, *BAX* means expression levels in transfected HL-60 cell lines were considerably lower than those in untransfected ones (8.21.3 vs. 1.43.01, P=0.01), but *MCL-1* and *BCL-2* mean expression levels in the transfected group were higher than those in the untransfected group, although these differences were not statistically significant (0.4±1.8 vs. 8.4±2.6; P=0.2 for *MCL-1* and 3.7±1.7 vs. 6.7±1.9; P=0.2 for *BCL-2*, respectively, Fig. 2A).

BAX, BCL-2 and MCL-1 Expression after LNA-anti-miR-222 Transfection

The data revealed that in the case of cell transfection with LNA-anti-miR-222, the mean expression level of *BAX* was significantly increased in the transfected group compared to non-transfected one (-2.1±2.2 vs. 6.4±2.3, respectively; P=0.01), while, the mean expression of *BCL-2* was significantly decreased in the transfected group compared to the non-transfected (3.3±1.4 vs. -1.8±1.4; P=0.01). Moreover, no statistically significant decrease was observed for the *MCL-1* gene (3.2±1.4 vs. -0.2±1.4; P=0.1, Fig. 2B).

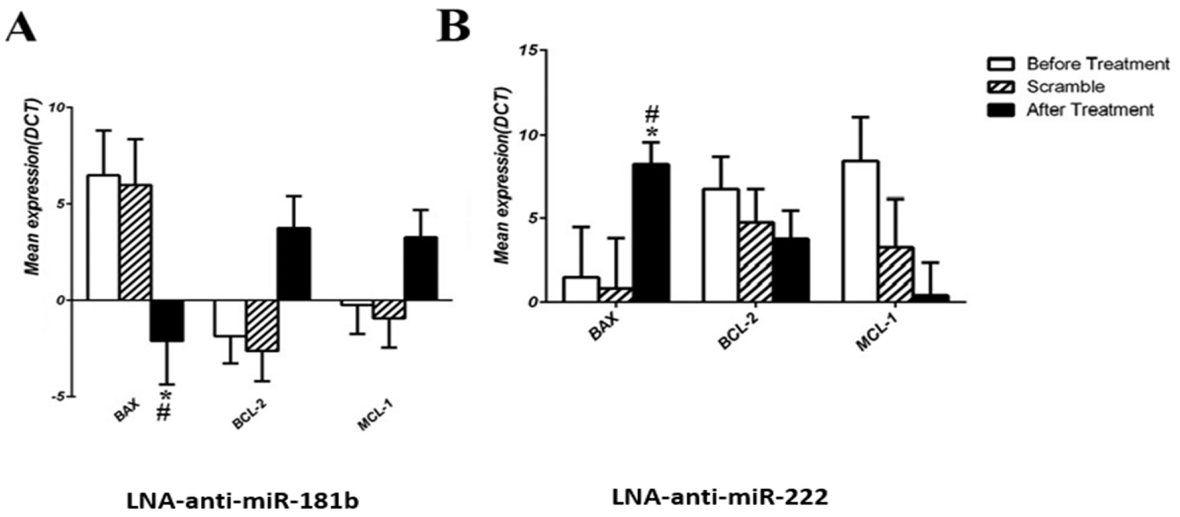

Figure 2: Changes in *BAX*, *BCL-2*, and *MCL-1* gene expression following miR-181b blockage with LNA-anti-miR (**A**), and following miR-222 blockage with LNA-anti-miR (**B**).
*P< 0.05 vs non-transfected; #P< 0.05 vs scrambled LNA.

The LNA-anti-miRs (miR181b and miR-222) could Affect Apoptosis

After transfection with LNA-anti-miRs, the HL-60 cell line's annexin $V^+/7\text{-}AAD^+$ was analyzed (Fig. 3A). Following gating, normal cells that are deemed viable are PE Annexin V and 7-AAD negative. Dead cells were scored as necrotic (annexin $V^-/7\text{-}AAD^+$, upper left quadrants, Q1), late apoptotic (annexin $V^+/7\text{-}AAD^+$, upper right quadrants, Q2), or early apoptotic (annexin $V^+/7\text{-}AAD^-$, lower right quadrants, Q (lower left quadrants, Q4). Using flow cytometry, the effects of LNA-anti-miRs (miR181b and miR-222) on apoptosis in the HL-60 cell line were investigated (Fig. 3B). The total percentage of overall apoptosis was calculated as the sum of early and late apoptosis percentages. The data come from three separate investigations. In line with these findings, flow cytometry demonstrated that LNA-anti-miRs (miR181b and miR-222) dramatically altered the HL-60 cell line's apoptosis (P<0.05) when compared to the control (HL-60 cell that was transfected by scramble). For LNA-anti-miR-222 and LNA-anti-miR-181b, the transfection demonstrated an apoptosis percentage of 24.29% and 6.41%, respectively. Additionally, a scrambling had an 11.4% apoptotic rate (Fig. 3A and 3B). These findings demonstrated that LNA-anti-miR-222 enhanced HL-60 cell mortality (P<0.0001). However, LNA-anti-miR-181b caused HL-60 cells to undergo less apoptosis (P=0.001).

WT1, C-KIT, and CEBPA Expression after LNA-anti-miRNA Transfection

The expression of the genes for *WT1*, *C-KIT*, and *CEBPA* was examined to determine whether blocking miR-222 and miR-181b can alter the expression of *WT1*, *C-KIT*, and *CEBPA* after successfully blocking the miR-181b and miR-222a by transfection of HL-60 cell lines with LNA-anti-miRNA. The results demonstrated that mean *C-KIT* expression in transfected HL-60 cell lines with LNA-anti-miR222 was significantly reduced compared to non-transfected one (5.3.2.2 vs. 7.23.7, respectively; P=0.03), whereas mean *WT-1* and *CEBPA* expression did not change significantly in a transfected group compared to non-transfected one (1.51.3 vs. 3.41.8, respectively; P=0.3 and (Fig. 3C). Contrarily, when cells were transfected with LNA-anti-miR-181b, the mean expression of *C-KIT* increased significantly in comparison to the non-transfected group (3.82.1 vs. 2.31.6, respectively; P=0.04), but *WT-1* and *CEBPA* did not change significantly in the transfected group compared to the non-transfected group (5.21.6 vs. 3.22.3, respectively; P=0.2 and 6.9 (Fig. 3C).

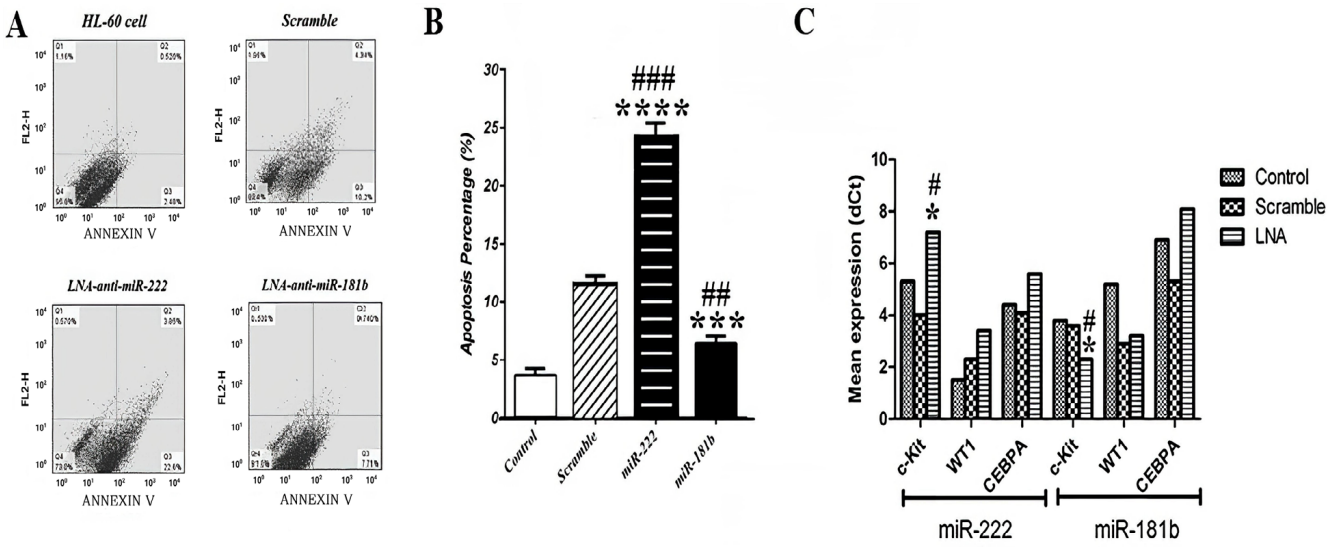

Figure 3: The flow cytometry results of apoptosis induction following miR181b and miR-222 blockage with LNA-anti-miR (**A**), the proportion of cells that are Annexin V-positive (B), and modification in *C-KIT*, *WT1*, and *CEBPA* gene expression after LNA-anti-miR blocked miR-222 and miR-181b.
*P< 0.05, ***P< 0.001, ****P≤ 0.0001 vs non-transfected; #P< 0.05, ##P≤ 0.01, ###P< 0.001 vs scrambled LNA.

DISCUSSION

Recent research indicates that microRNAs have a significant role in the etiology of human cancers including hematologic malignancies like AML. These compounds can function as tumor suppressors or oncogenes. Investigation of these molecules may provide a promising and novel epigenetic targeting treatment approach in combination with conventional chemotherapy in AML patients. The miR-181b and miR-222 are essential factors in the apoptotic process [41, 43]. They both are considered as apoptosis [51, 52].

In the present work, we sought to identify miR-222 and miR-181b expression as novel molecular biomarkers in AML patients. We also assessed the impact of miR-222 and miR-181b blockade on the expression of the *WT1*, *CEBPA*, and *C-KIT* genes, which are typically dysregulated in AML patients, for the first time based on our understanding.

Several reports indicated the miR-222 tumorigenesis, as it was overexpressed in AML [35]. We found that cell viability was remarkably decreased in the LNA-anti-miR-222 transfected cells as it led to increased apoptosis. We observed a significant increase of *BAX* expression in LNA-anti-miR-222 cells which is a pro-apoptotic member of the Bcl-2 family and regulates the intrinsic apoptotic pathway [53]. *BCL-2*, however, was significantly decreased which has an anti-apoptotic characteristic in the BCL-2 family. Based on this evidence, silencing miR-222 which has oncogenic characteristics may represent a successful therapeutic strategy for AML patients as it leads to increased cancer cell apoptosis through the intrinsic apoptotic pathway.

In AML patients, miR-181 expression is linked to a better prognosis [14, 54]. The previous study has shown that over-expression of miR181b leads to modulating hematopoietic lineage differentiation and enhances apoptosis significantly [55-59]. We discovered that miR-181b blockade greatly enhanced cell viability, as LNA-anti-miR-181b led to reducing the apoptosis of HL-60 cells. In line with our study, other studies also showed the effective role of miR181b in apoptosis through the employing of certain mediators such as TGF-β and NF-κB signaling pathways [60, 61]. We also observed the significant up-regulation of the anti-apoptotic gene, *BCL-2*, and down-regulation of the pro-apoptotic *BAX* gene in the transfected HL-60 cell line with LNA-anti-miR181b. Up-regulation of *BCL-2* which has anti-apoptotic characteristics follow-

ing the inhibition of miR181b, suggests that miR181b induces apoptosis and causes a favorable outcome. In a study by Kronski et al, they also found that over-expression of miR181b in MDA-MB-231 cells could double the apoptosis rate [62]. Consistent with our study, other studies have shown that *BCL-2* and *MCL-1* with anti-apoptotic features are the targets of miR-181a/b, and they lead to apoptosis resistance in leukemia [63]. It was recently found that up-regulation of MiR-181a and miR-181b, in lymphoma have along with better prognosis [64], as miR-181b enhances differentiation, maturation, and immunosuppression. By increasing IL-10 and TGF-β, for instance, and decreasing circulating TNF-α and IL-6, it improves the activities of many innate and adaptive immune cells, creating an autocrine loop that intensifies immunosuppression [65].

60–80% of AML patients have high levels of *C-KIT* expression [66]. The *C-KIT* oncogene encodes the class III transmembrane receptor tyrosine kinase, often known as CD117 [67]. In less than 5% of marrow myeloid progenitor cells is it expressed [68]. 70% of AML patients' blasts display *C-KIT* expression [69]. Overexpression of both wild and mutant forms of *C-KIT* has been shown to encourage leukemogenesis [67]. While its down-regulation can stop hematological malignant cells from growing or spreading [70]. In our study, we showed that the expression level of *C-KIT* in transfected LNA-anti-miR222 cells was significantly decreased, which is along with a better prognosis. However, an increase in the *C-KIT* expression level in LNA-anti-miR-181b transfected cells was observed, and based on the previous studies its overexpression can lead to abnormal cell proliferation and poor outcomes [54, 71]. So based on the critical role of c-KIT signaling, targeting this molecule could be a novel therapeutic approach for the treatment of cancer, especially hematologic malignancies [72].

In conclusion, we show that miR-181b and miR-222 have potential therapeutic effects on AML cells. This article identifies a crucial area of research for examining whether miRNAs can increase the effectiveness of conventional therapy strategies. By influencing *BCL-2* and *BAX*, we discovered that down-regulating miR-222 in HL-60 cells increased the rate of apoptosis; however, inhibiting miR-181b had the reverse effect. We also demonstrated that the expression of the *C-KIT* oncogene can be influenced by miR-181b and miR-222. Inhibiting miR-222 and increasing miR-181b could be a novel therapeutic approach for treating AML that could be used alone or in combination with current therapies to overcome the current limitations in treating this malignancy. These two miRNAs can be used as biomarkers to monitor the response to the treatment of AML patients. In later trials, biological therapy can also be accomplished by focusing on the two miRNAs. The relevance of these microRNAs in AML has to be studied further by developing a transgenic mouse model. As a result, this study offers AML patients a tempting therapy strategy.

In conclusion, combining standard chemotherapy with regulating miR-222 and miR-181b expression may be a helpful strategy to reduce blast cell survival and leukemic cell proliferation in AML patients. Additionally, we demonstrated for the first time that the *C-KIT* gene may be a new target for miR-222 and miR-181b.

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