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THE EFFECT OF INHIBITION OF LNCRNA MIR100HG ON THE PROLIFERATION OF HUMAN PROMYELOCYTIC LEUKEMIA CELLS

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ABSTRACT

lncRNAs are one of the main classes of non-coding RNAs, responsible for RNA regulation in a variety of cellular processes, and their effect on human cancer remains largely unexplored; Abnormal expression of lncRNAs has been shown to be associated with many human diseases and cancers, such as leukemia. Acute promyelocytic leukemia (APL) is the M3 subtype of acute myeloid leukemia (AML), with the aberrant accumulation of promyelocytes. One of the lncRNAs that showed upregulation in AML is lncRNA MIR100HG, involved in different cancers. The aim of our study is to investigate the functional role of MIR100HG antisense LNA GapmeRs, on APL cells. In this experimental study**,** we have used an Antisense LNA GapmeRs, in order to block MIR100HG in APL Cells. HL60 (APL cell line) cells were transfected with MIR100HG antisense LNA GapmeRs and at three different time points (24, 48 and 72 h) and were investigated apoptosis, necrosis, MIR100HG and TGFβ expression. MIR100HG inhibition could reduce the viability of HL- 60 cells, through induction of apoptosis; because of the TGFβ upregulation. qRT-PCR was performed to determine the MIR100HG expression by antisense LNA GapmeRs. Our results suggest that degradation of MIR100HG could serve as a novel approach for controlling the proliferation of APL cells and therefore, can be used in translational medicine for targeted therapy in APL.

KEYWORDS: ***LncRNA MIR100HG; Antisense LNA GapmeRs; TGFβ; Acute promyelocytic leukemia***

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INTRODUCTION

Non-coding RNAs (ncRNAs) are divided into two subclasses, according to the size of the translated fragment. ncRNAs smaller than 200 nucleotides (nt) are called small or short non-coding RNAs, whereas lncRNAs are more than 200 nt in length, and are rather poorly understood and often dismissed as only transcriptional 'evolutionary junk' [1-3]. The expression of lncRNAs seems to be strictly regulated, in physiological conditions, as well as in several human diseases, including cancer. More broadly, they modulate transcriptional regulation, regulate protein activities, and play structural or organizational roles. In addition, they play critical roles in tumorigenesis, including cell cycle progression, apoptosis, and metastasis [4-7]. APL, is the M3 subtype of AML [4], with an aberrant accumulation of promyelocytes, as a hallmark. APL is distinguished by a balanced reciprocal translocation between chromosomes 15 and 17, prompting the expression of the recombinant protein PML-RARα [1, 2, 5, 6, 8]. Overexpression of some lncRNAs may function as an oncogene, through the negative regulation of tumor suppressor genes. Many lncRNAs have already been shown to serve as biomarkers or therapeutic targets, for many diseases, cancer diagnosis and prognosis [1, 6, 9]. They have also been utilized as therapeutic targets for selective killing of cancer cells [9]. The role of lncRNAs in a number of cancers is evident. For example, HOX antisense intergenic RNA myeloid 1 (HOTAIRM1), transcription factor PU.1 and PVT1 are highly expressed in APL [5, 8]. The LincRNA MIR100HG gene is located on human chromosome 11q24.1, in the mir-100-let-7a-2/miR-125b-1 cluster and it is known that this lncRNA has an important role during hematopoiesis and the pathogenesis of acute megakaryoblastic leukemia (AMKL) [10]. Intergenic lncRNAs (lincRNAs) are located between protein-coding genes and are transcribed, independently [1]. The participation of MIR100HG was indicated in the gap junction pathway and TGF-β pathway also has a probable role in regulation of lymph node metastasis in the early-stage of cervical cancer [11]. GapmeR has emerged as a new class of molecule that includes the locked nucleic acid (LNA)-conjugated chimeric single-strand antisense oligonucleotide, with the ability to knock down a target gene of interest with precise specificity, through the post-transcriptional gene silencing [12]. The combination of both chemistry and structural modifications provide GapmeR with high binding affinity for the target mRNA and confers increased resistance to endo- and exonucleases, low levels of toxicity and improved stability in biological serum and cell culture medium [13]. When GapmeR molecule enters the cell, GapmeR-mRNA duplex will be formed by the central antisense DNA in the gap, bind to the endogenous mRNA. Subsequently, the GapmeR-mRNA duplex is recognized by the cellular enzyme RNase H, degrading the targeted mRNA and thus inhibits the specific gene [14]. The purpose of this study was to block MIR100HG and evaluate the effect of blocking on cell proliferation, apoptosis, necrosis and TGFβ expression level in promyelocytic cells (HL 60), using Antisense LNA GapmeRs technology.

MATERIALS AND METHODS

In this experimental study, we investigated the functional role of MIR100HG antisense LNA GapmeRs, on APL cells.

Cell culture

The HL-60 cell line (Human APL) was purchased from the National Cell Bank of Iran (Pasteur Institute, Tehran, Iran). The cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 (Gibco, Paisley, UK) medium, supplemented with 15% (v v^{-1}) fetal bovine serum (Gibco, Paisley, UK), 100 U ml^{-1} of penicillin and 100 g ml^{-1} of streptomycin (Sigma-Aldrich, Saint Louis, MO, USA), in a 25-cm^2 culture flask (Nunc, Roskilde, Denmark), and incubated with 5% CO_2 at 37 °C. To maintain the exponential phase, cells were passaged two times per week.

Cell transfection

The accession number of MIR100HG was obtained from www.ensembl.org, as ENSG00000255248. Antisense LNA GapmeRs sequence 5′ATCGATTGGTTAGTGT 3′ and Antisense LNA GapmeRs Negative Control (ALGNC) (scrambled) sequence 5′AACACGTCTATACGC 3′ for hsa-mir-100-let-7a-2, were purchased from the Exiqon (Copenhagen, Denmark). LNA GapmeRs and ALGNC were labeled at their 5′ ends with a fluorescent dye, 6-FAM (6-carboxyfluorescein). For HL60 cell transfection, the Polyfect Transfection Reagent (Qiagen, Hilden, Germany) was used, according to the company's guidelines. A total number of 5×10^5 HL60 cells in the exponential phase, were cultured in six-well culture plates (Nunc), encompassed with 1.8 ml of RPMI-1640 medium per well, with no antibiotics and fetal

bovine serum. Six picomoles of antisense LNA GapmeRs lncRNA inhibitor was mixed with 12 µl of Polyfect Transfection Reagent, in a final volume of 200 µl of Opti-MEM Medium (Gibco, Paisley, UK), and subsequently incubated at room temperature for 15 min. Then, the complex was added to the cells and swirled cautiously, to ensure even distribution over the entire plate surface. After 6 h incubation, fetal bovine serum and antibiotics were added to the cells and then incubated for 24, 48 and 72 hours. The transfected cells and untreated cells were cultured in parallel to the antisense LNA GapmeRs lncRNA-transfected cells with ALGNC. The transfection was examined, using fluorescence microscopy and flow cytometry. As LNA-anti-lncRNA was conjugated by 6-FAM, HL60-transfected cells with Antisense LNA GapmeRs were detected, using fluorescence microscopy and flow cytometry.

Reverse transcriptase lncRNA real-time polymerase chain reaction

The expression level of MIR100HG after an Antisense LNA GapmeRs transfection was determined by reverse transcriptase (RT) lncRNA quantitative real-time PCR (ExiLERATE LNA™ qPCR). The total RNA was extracted, using the miRCURY RNA Isolation Kit (Exiqon) at 24, 48 and 72 h after transfection, and then cDNA was synthesized, using ExiLERATE LNA™ qPCR, cDNA Synthesis kit (Exiqon). ExiLERATE LNA™ qPCR, SYBR® Green master mix kit and ExiLERATE LNA™ qPCR specific MIR100HG primers, purchased from Exiqon were used for real-time PCR. Synthetic RNA spike-in templates and their primers (Exiqon, Copenhagen, Denmark) were used, as real-time PCR internal control, following the manufacturer's protocol, using the following primers for *MIR100HG*: Forward; 5'TGCTCGTTCCTGTTGTGTTC 3' Reverse; 5' AGGAGGTGAACGATTGGATG3'. *MIR100HG* expression level in each sample was normalized to *GAPDH* expression level, using the following primers: forward: 5'GGTGTGAACCATGAGAAGTATGA 3', reverse: 5' GAGTCCTTCCACGATACCAAAG 3'. The reaction was performed in the following condition: preliminary denaturation at 95°C for 10 min, followed by 45 cycles of 95°C for 10 s and 60 °C for 1 min. StepOnePlus Real-Time PCR Systems (ABI Applied Biosystems, Foster City, CA, USA) and ΔΔCt method were used for qPCR tests and data analysis.

Assessment of TGFβ expression level with real-time qPCR

The *TGFβ* expression level was determined by real-time qPCR. The miRCURY RNA Isolation Kit and ExiLERATE LNA™ qPCR, the cDNA Synthesis kit from Exiqon were used for total RNA extraction and cDNA synthesis. The ExiLERATE LNA™ qPCR, SYBR® Green master mix kit (Exiqon) was used for *TGFβ* real-time PCR and *GAPDH* acted, as an internal control. The primer sequences for *TGFβ* were as follows: forward; CTCGCCAGAGTGGTTATCTT and reverse; GTAGTGAACCCGTTGATGTC. The StepOneplus™ Real Time PCR systems (ABI Applied Biosystems, USA) instrument and the ΔΔCt method were used for qPCR experiments and data calculation, respectively.

Cell viability assay

The HL-60 Cell viability was assessed, using the MTT (3-[4,5 dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) (Sigma-Aldrich, Saint Louis, USA) assay, based on the reduction of MTT by the mitochondrial dehydrogenase of intact living cells, resulting in a purple formazan product. This modification is directly related to the number of living cells. The MTT assay was completed at three time points 24, 48 and 72 h after transfection. Subsequently, 200 µl of MTT (Sigma- Aldrich) with 50 mg/ml concentration was added to the 5×10^5 HL-60 cells, suspended in 2 ml of RPMI-1640 medium, and then incubated for 6 h at 37°C in darkness. After primary steps, 200 µl of dimethyl sulfoxide (DMSO) (Sigma-Aldrich) was added to each well and shaken until the crystals are dissolved. Blank samples were prepared, using the same method. The optical density was detected at 570 nm, using a spectrophotometer (PG Instrument T80, Leicestershire, England). Reading was transformed to the percentage of the controls.

Apoptosis and necrosis assay

The FITC/Annexin-V Apoptosis Detection Kit with PI (Biolegend, San Diego, USA) was used for detection of apoptosis and necrosis. Annexin-V was used for identification of apoptotic cells, and propidium iodide used for differentiation of necrotic cells. The cells, seeded at a density of 5×10^5 HL-60 cells per well, transferred to flow cytometry tubes and centrifuged for 5 min at 1500 rpm. The supernatant was removed, and cells were washed with 1 ml of cold phosphate-buffered saline (Gibco, Paisley, UK). One hundred milliliter of the prepared solution (based on kit instruction) was

added to each tube and incubated in the dark at RT for 15 min. After incubation, 300 μl of annexin-V binding buffer solution was added to each tube and analyzed by FACS Calibur flow cytometer (BD, California, USA) with 488 nm excitation, 515 nm band-pass filter for fluorescein-conjugated annexin-V detection, and a filter>600 nm for PI detection.

STATISTICAL ANALYSIS

All tests were performed in triplicate, and analyzed with SPSS (version 22) software (IBM, New York, NY, USA). Our results were analyzed with two-way analysis of variance (ANOVA). The data were represented as mean ± SD. Statistical significance was defined as $p < 0.01$.

RESULTS

Antisense LNA lncRNA inhibitor strongly inhibit MIR100HG

The transfection efficiency of HL60 cells, transfected with antisense LNA GapmeRs was about 80% (Fig. 1). We have used specific GapmeR molecules to block the expression level of MIR100HG by reverse transcriptase-quantitative PCR. HL60-cells were transfected with the miRCURY LNA lncRNA inhibitor, which can lead to effective gene silencing. For this purpose, we designed and synthesized a panel of specific GapmeR molecules targeted cells, transfected with antisense LNA GapmeRs (LNA GapmeRs group). The control was transfected with ALGNC and untreated HL60 cells used as untreated groups. QRT-PCR was applied at 24, 48 and 72 h after transfection. There was no statistically significant difference between values of MIR100HG in untreated groups and ALGNC groups. Expression of MIR100HG was significantly lower in the antisense LNA GapmeRs group at all three-time points, compared with the untreated group ($p < 0.001$), (Fig. 2).

GapmeR molecules by inhibiting MIR100HG, decrease the cell viability in promyelocytic leukemia cells

Cell viability and cytotoxicity of HL60 cells were examined by MTT cell assay kit, as per manufacturer's instructions. Yellow colored water-soluble tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was reduced to formazan crystals and then the crystals were dissolved in DMSO. Three thousand cells per well were seeded in 96-well plates, 24, 48 and 72 hours after transfection. After 72 h incubation, cells were treated by antisense LNA GapmeRs on HL60 cells, the cell viability was significantly decreased to 58%. The absorbance was recorded at 570 nm on a microplate reader (BioRad, USA). The difference in cell viability at all three time points was significant in the antisense LNA GapmeRs group, ALGNC group and the untreated groups ($p < 0.001$, Fig. 3).

GapmeR molecules by inhibiting MIR100HG, induce apoptosis and necrosis in promyelocytic leukemia cells

To validate the cell death by apoptosis, annexin V-FITC/PI double staining was performed in HL60 cells, at three different time points, after transfection. Due to the toxicity of transfection reagent in GapmeRs treated group, the apoptotic ratio in ALGNC group was slightly more than the untreated group; although the values were not statistically important. A gradual increase in apoptosis of HL60 cells at 24, 48 and 72 h after transfection with antisense LNA GapmeRs were comparable with ALGNC transfected cells and untreated cells. The highest amount of apoptosis was observed at 72 h post-transfection. Above all, it seems MIR100HG inhibition is associated with an increase of apoptosis in HL60 cells. In the antisense LNA GapmeRs group, the apoptotic ratio was increased, compared with the control groups at three-time points 24, 48 and 72 h after transfection ($p < 0.001$); Fig. 4). The percentage of early apoptotic HL60 cells minimally increased, at 24 h post-transfection. Furthermore, at all three-time points in HL60 cells, the late apoptotic ratio confirmed to be associated with the MIR100HG inhibition, because of an increase in the antisense LNA GapmeRs group, compared to the other groups. ($p < 0.001$); Fig. 4).

The TGFβ expression level increased in promyelocytic leukemia cells, after inhibition with MIR100HG

Finally, to examine the functional influence of GapmeR-mediated gene silencing of TGFβ, we performed qRT-PCR on LNA GapmeRs, ALGNC and untreated groups in HL60 cells, at 24, 48 and 72 h after transfection. GAPDH was used as an internal control for normalization. However, TGFβ relative expression showed notably the difference between LNA GapmeRs, ALGNC and the untreated groups ($p < 0.001$). The highest level of TGFβ relative expression was at 72 h after transfection in HL60 cells (Fig. 5).

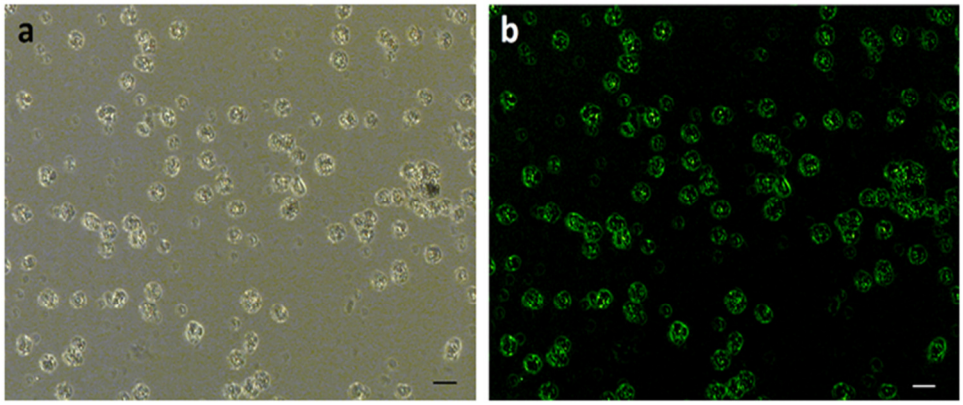

Figure 1

HL60 cells were transfected with 6-FAM labeled antisense LNA GapmeRs, and the transfection efficiency was evaluated by fluorescent microscopy. Phase contrast (a) and fluorescent (b) images of the same field of HL60 cells display that the majority of cells are transfected. Scale bars: 50 µm.

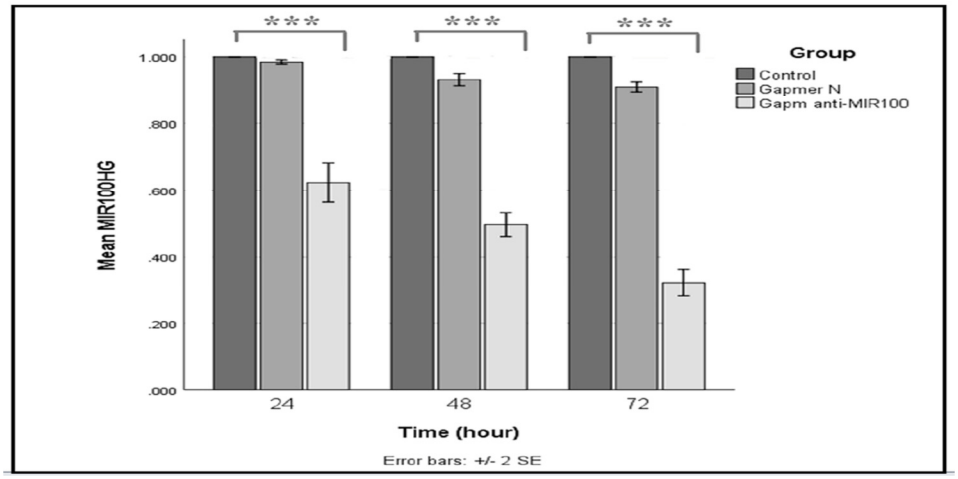

Figure 2

The MIR100HG expression level was determined, using qRT-PCR assay, at 24, 48 and 72 h after transfection. Data analysis was performed, using ΔΔCt method. The untreated cells were used as a control group and as a reference for comparison with other groups. The data are presented as mean ± SD of three independent experiments. *** p < 0.001

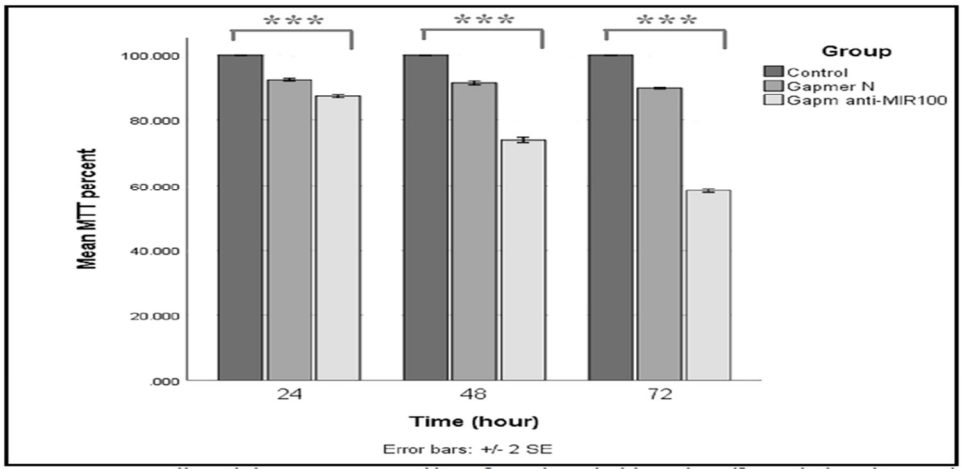

Figure 3

HL60 cells viability was measured by 3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay at 24, 48 and 72 h after transfection. The viability of the untreated cells at each time point was considered as 100% and the viability of other groups is displayed as a percentage of the untreated cells, at the same time point. The data are presented as mean ±SD of three independent experiments. *** p < 0.001

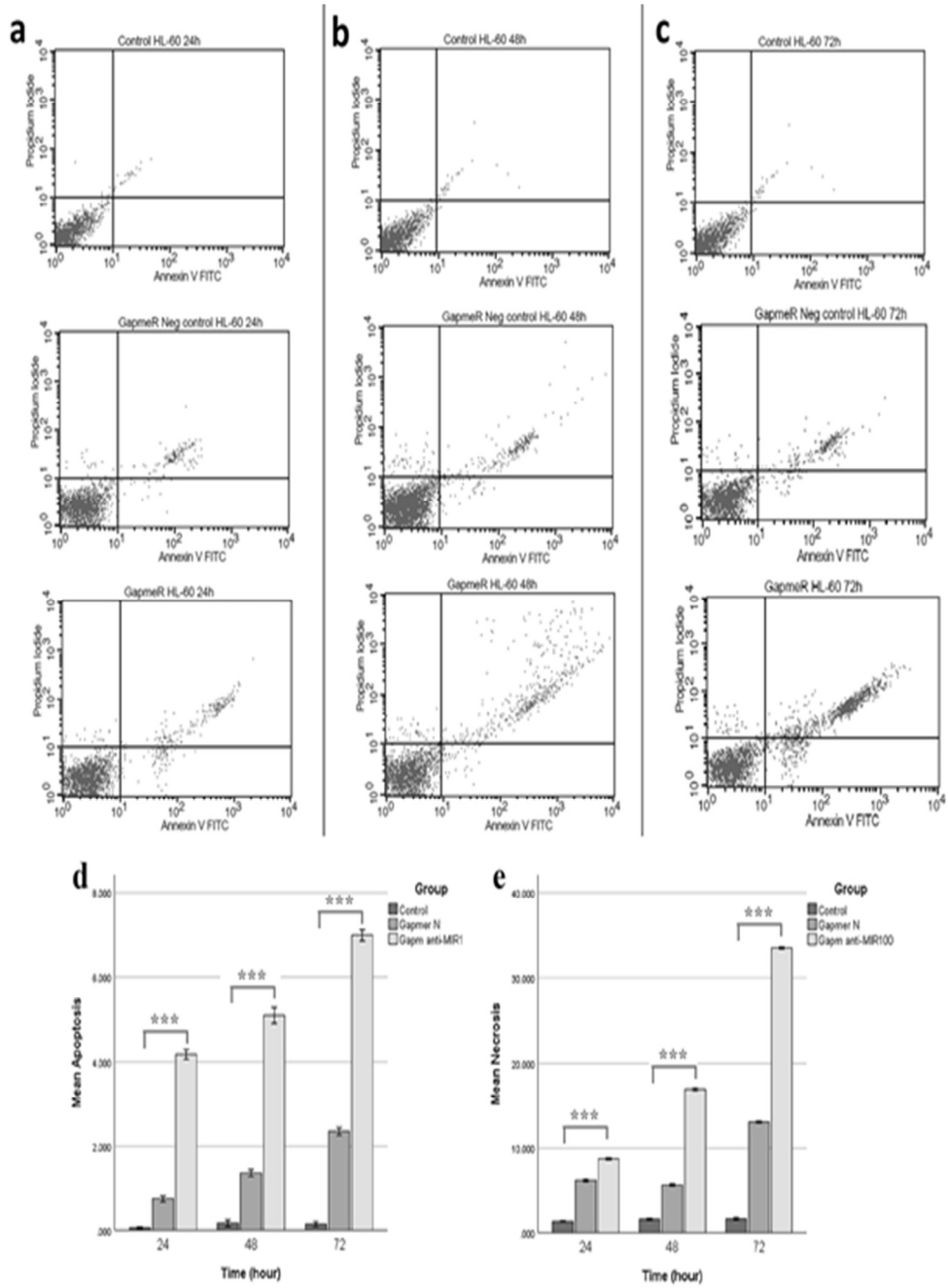

Figure 4

HL60 cells apoptosis and necrosis were evaluated by annexin V–propidium iodide staining at 24, 48 and 72 h after transfection. Flow cytometry analysis was performed, using 488- nm excitation and a 518 -nm band-pass filter for fluorescence detection, and a filter of 617 nm was used for the PI detection. Representative cytofluorimetric graphs are shown in (a-c). The ratio of apoptotic cells was increased by MIR100HG antisense LNA GapmeRs transfection, at three time points. Data shown in the graph are presented as mean ± SD of three independent experiments ($p < 0.001$) (d). The ratio of necrotic cells was increased by MIR100HG antisense LNA GapmeRs transfection at the three-time points. The data shown in the graph are presented as mean ± SD of three independent tests (*** $p < 0.001$) (e).***

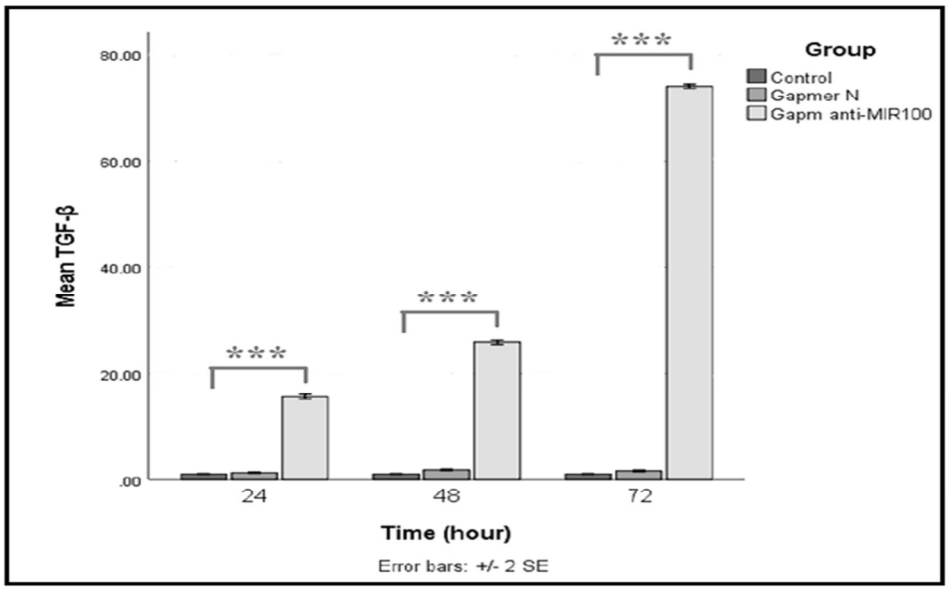

Figure 5

The expression level of TGFβ was determined using qRT-PCR assay at 24, 48 and 72 h after transfection. The ΔΔCt method was used for data analysis and untreated cells were considered, as a control group and as a reference for comparison with other groups. The data are presented as mean ± SD of three independent experiments. *** p < 0.001

DISCUSSION

A routine approach for AML treatment is chemotherapy [15], however, this method is affected by the resistance that decreases the treatment efficiency in many cases, as it is frequently exemplified by resistance to ATRA [16, 17]. In addition, the combination of cancer cells, and oncogenic lncRNA inhibitors, are more sensitive to chemotherapy agents, compared to each alone [18]. Furthermore, ATRA is a routine drug that is used for treating APL, but it is not always successful [17]. This study suggests that Anti lncRNA MIR100HG can be used with or without ATRA for treating APL, which is resistant to chemical treatment. *LncRNA MIR100HG involves in progression of myeloid leukemia, by regulating hematopoiesis and oncogenes, and it is located on chromosome 11q24.1*[10]. In a GSEA (Gene Set Enrichment Analysis) study, Shang . have identified 3,489 genes. Among those, 1,127 and 2,362 gene sets are positively or negatively associated with MIR100HG, respectively, using the entire mRNA expression dataset and the MIR100HG expression level [11]. In AMKL, the lncRNA MIR100HG is among the highly expressed non-coding RNA. Also, MIR100HG has an important mediator role in the development and hematopoiesis in AMKL [11]. Furthermore, the intronic coding region in MIR100HG gene, acting as a proapoptotic molecule, induces apoptosis through caspase-dependent mitochondrial signaling pathway [19]. In addition, MIR100HG is associated with TGFβ signaling pathways and the gap junction. Nearly all of mRNAs co-expressed with MIR100HG, are involved in gap junction pathway [10, 11]. The TGFβ and Wnt pathways counterbalance silencing and differentiation, HSC self-renewal and proliferation, as major regulatory signaling pathways [20]. The hematopoietic stem cells are established by TGFβ, by induction of apoptosis and growth arrest, in early HSPCs, while HSCs are in quiescence [21, 22]. Contrary to TGFβ, megakaryopoiesis and self-renewal in HSCs are positively regulated by Wnt signaling [23-25]. Finally, we have shown that target-specific gene silencing can be induced, by specifically designed chimeric GapmeR molecules. A study shows that in leukemic cells, GapmeR can be internalized by micropinocytosis and mediate post-transcriptional gene silencing, which can be used as a potent and non-invasive method. GapmeR molecules, causes RNaseH-mediated gene silencing, independent of RNA-Induced Silencing Complex (RISC), and therefore, it does not cause RISC-associated or microRNA-like off-target activity. Because GapmeRs are short (13–20 nucleotides), off-target and mismatches have rarely occurred [14, 26, 27]. In the present study, we have been using antisense LNA GapmeRs to knock down lncRNA MIR100HG in acute promyelocytic

leukemia (AML-M3) cell line (HL60). Additionally, we showed the evidence for the first time that upregulated MIR100HG expression is involved in the proliferation of primary APL cells. Real-time PCR data confirmed that this lncRNA was mostly downregulated after antisense LNA GapmeRs transfection. The apoptosis/necrosis assay showed that antisense LNA GapmeR MIR100HG transfection significantly increased apoptosis and necrosis. In these assays, the effect of antisense LNA GapmeR MIR100HG transfection on cell viability was much higher than in the basal transfection reagent toxicity, observed with ALGNC oligonucleotide transfection. Furthermore, it was shown that reduction in cell viability was associated with the inhibition of lncRNA MIR100HG, using the MTT method. Although HL60 cell viability was reduced slightly in the ALGNC group, compared with the untreated group, which is probably due to the toxicity of the reagents used in the transfection; however, this reduction was not statistically significant. Conversely, TGFβ expression level was increased, in the antisense LNA GapmeRs group. In summary, the results of the present study suggested that inhibition of MIR100HG with antisense LNA GapmeRs is a potential drug-based antisense therapy in APL, which can be used individually or in combination with chemical conventional therapies, in case of resistance to treatment. In addition, further *in vivo* studies has to be performed that might help to avoid an off-target knockdown, as new possibilities for using GapmeRs as an effective therapeutic tool with accuracy in biological function, before administration in clinical trials.

CONCLUSION

Collectively, we identified that the lncRNA MIR100HG degradation, using antisense LNA GapmeRs, induces apoptosis, necrosis and reduces cell proliferation in HL60 cell. Thus, inhibition of MIR100HG might represent as potential strategies for controlling APL proliferation and may be used individually or combined with current therapies, for AML treatment. In the future studies, the diagnostic and prognostic performance of MIR100HG should be evaluated with existing clinicopathological and serological markers in larger cohorts, in order to accelerate the clinical utilization.

Compliance with ethical standards

Ethical approval

The local ethics committee of Isfahan University of Medical Sciences (IRAN) approved this study, and the studies have been approved by the appropriate institutional and/or national research ethics committee and have been performed in accordance with the ethical standards, as laid down in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

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CONFLICT OF INTEREST

Conflict of interest declared none.

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