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Effects of hsa-piR-32877 Suppression with Antisense LNA GapmeRs on the Proliferation and Apoptosis of Human Acute Myeloid Leukemia Cells

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ABSTRACT

Acute myeloid leukemia (AML) is an invasive form of hematologic malignancies which results in the overproduction of myeloid cells in the bone marrow. Aberrant expression of piwi-interacting RNAs (piRNAs) which belong to small non-coding RNAs, play important roles in different cancer cells' progress. hsa- piR- 32877 is up-regulated in AML. Down regulation of hsa-piR-32877 by antisense LNA GapmeRs could be potential for suppression of myeloid cell proliferation and induce myeloid cell apoptosis. We have blocked the expression of hsa-piR-32877 by antisense LNA GapmeRs in human bone marrow blast cells, and the M-07e cell line. Samples were transfected with antisense LNA GapmeRs at 24, 48, and 72 hours. The Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was performed to investigate the expression of hsa-piR-32877, CASP3, and CASP9. Both CASP3 and CASP9 play important roles in apoptosis. Cell proliferation was studied via CFSE (carboxyfluorescein diacetate succinimidyl ester) assay. Results showed that hsa-piR-32877 was down-regulated by antisense LNA GapmeRs in the patient and cell line samples. Also, after transfection, cell proliferation and apoptosis decreased and increased, respectively. Our data suggested that hsa-piR-32877 suppression may act as a novel therapeutic method for the inhibition of human leukemic cells proliferation in AML.

Keywords: Acute myeloid leukemia, Antisense LNA GapmeRs, hsa-piR-32877, *CASP3*, *CASP9*

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Introduction

Acute myeloid leukemia is an invasive form of hematologic malignancies, characterized by an increase in the number of myeloid cells in the bone marrow and differentiation arrest of myeloid progenitor cells (1,2). Based on the French–American–British (FAB) system, there are eight types of AML (M0–M7), which are different in morphological characteristics and the degree of leukemic-cell differentiation (3). Despite chemotherapy and other treatments, a significant proportion of patients die of their disease. Also, most patients with AML will relapse after treatments (4-6). Therefore, there is a need for developing novel and potential treatments.

Piwi-interacting RNAs are a class of small non-coding RNAs that possess 24-31 nucleotides in length (7, 8). PIWI (p-element-induced wimpy testis) is a subfamily of Argonaute proteins- Argonaute proteins are highly conserved between species- which piRNA binds to PIWI resulting in regulatory roles in the cells (9, 10). piRNAs are 2'-O-methylated at their 3' ends, so they are capable of binding to PIWI proteins (11). piRNAs are transcribed from RNA polymerase II and undergo 5′ end-capping, 3′ end polyadenylation, and sometimes selective splicing occurs (12). Previous studies show that piRNAs are involved in transposon silencing, transcriptional gene silencing (TGS), post-transcriptional gene silencing (PTGS), and epigenetic regulation of gene expression (13, 14). Current evidence shows that piRNA- PIWI complexes are abnormally expressed in many cancers and play key roles in cancer progress and metastasis (15, 16). Among piRNAs, it has been studied that piR-32877 is up-regulated in AML patients, so it could be a novel diagnostic biomarker in AML (17).

GapmeRs are antisense oligonucleotides flanked by LNA-modified oligonucleotides, inducing target RNA knockdown through intracellular ribonuclease H1 (RNAseH1). Locked Nucleic Acids (LNAs) are RNA analogs, because of the bridge that connects 2′-oxygen to 4′-carbon in the sugar structure, this group of nucleic acid analog is called locked nucleic acid, leading to a closed ribose group in the C3′-endo. The 2′-O-CH2-4′ linkage converts a flexible furanose into a rigid structure. LNA oligonucleotides increase target affinity, thermal stability, and nuclease resistance so enhanced the thermodynamic stability of GapmeRs (18-20).

In this study, we have used antisense LNA GapmeRs for inhibition of hsa-piR-32877 in a human AML cell line (M-07e) and AML patients; and analyzed its effect on cell proliferation and apoptosis.

Materials and methods

Specimens

Bone marrow samples from two patients with informed consent were collected in ethylenediamin-etetraacetic acid (EDTA) tubes from Sayed Al-Shohada Hospital (Isfahan, Iran). In this study, all of the patients did not receive any treatments. Besides, the human AML cell line M-07e was purchased from the National Cell Bank of Iran (Pasteur Institute, Iran).

Cell culture

Blast cells were isolated from bone marrow using Ficoll (Biogene, Iran). Five ml of normal saline (sodium chloride 0.9%) was added to each sample with a 5 ml volume. Then, 8 ml of blood that mixed with normal saline was transferred on 3 ml of Ficoll. Samples were centrifuged at 3000 RPM (revolutions per

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minute) for 20 min, and the cells were isolated. Equal to the volume of isolated cells, normal saline was added. Samples were centrifuged at 2500 RPM for 5 min. Then pellet was dissolved with the cell culture medium. Cells grow in T25 flasks including RPMI 1640 (BIO-IDEA, Iran) with 1 µl granulocyte-colony stimulating factor (G-CSF) (Filgrastim, Tinagrast, Iran) supplement, 10% fetal bovine serum (FBS) (Zisera, Iran), and 100 units/ml penicillin and streptomycin (BIO-IDEA, Iran). The cells were incubated at 37°C in 5% CO2.

CFSE assay

Flow cytometry was performed by CFSE Cell Division Tracker Kit (Biolegend, USA) for cell proliferation analysis. Excitation and emission of CFSE were analyzed with the BD FACSCalibur flow cytometer. A 5 mM solution was prepared by adding 36 µL of DMSO (dimethylsulfoxide) to 100 µg CFSE dye. Cells were centrifuged at 2500 RPM for 10 min. 1 mL of PBS was added to the cells pellet. Also, 1 µl of 5 mM CFSE stock solution was added to cells that resuspend with PBS. Cells were incubated for 20 min at room temperature. Five times the volume of original staining, the cell culture medium with 10% FBS was added. Samples were centrifuged at 2500 RPM for 10 min. Pellet cells were dissolved in a pre-warmed cell culture medium, and incubated for 10 min. Cells were centrifuged at 2500 RPM for 10 min. Pellet cells were ready for the next test (transfection).

Cell transfection

The hsa-piR-32877 sequence was downloaded from piRNAdb.hsa.v1_7_6 FASTA file (www. pirnadb. org). Antisense LNA GapmeRs and antisense LNA GapmeR negative controls for piR-32877 were purchased from Eurofins (Germany). Both antisense LNA GapmeRs and antisense LNA GapmeR negative controls possess a fluorescent dye called 6-FAM (6-carboxyfluorescein) at 5´ ends. Metafectene PRO (Biontex, Germany) was used for cell transfection. Cells were seeded into six-well culture plates that each well include 1.8 mL RPMI 1640 with no antibiotics and FBS. The amount of 24 µL of metafectene reagent was mixed with 600 µL of RPMI 1640. Then, 300 µL of this solution was added to vials that contain 6 µl of antisense LNA GapmeRs dissolved in 300 µL of RPMI 1640 and 6 µL of antisense LNA GapmeR negative controls dissolved in 300 µL of RPMI 1640, separately. Solutions were incubated at room temperature for 15 min. 200 µL of antisense LNA GapmeRs, and 200 µL of antisense LNA GapmeR negative controls were dissolved in the wells that related to antisense LNA GapmeRs and antisense LNA GapmeR negative controls, respectively. Besides, 200 µL of RPMI 1640 was added to the control well which was not transfected. Samples were incubated at 37°C in 5% CO2 for 4 hours. After incubation, 100 µL of FBS and 20 µL of antibiotics were added to the wells. The proliferation of the cells was investigated after 24, 48, and 72 hours with a flow cytometry assay. All of the processes were performed for patient samples and the cell line.

Quantitative real-time PCR (qRT-PCR)

RNAs were extracted from the cells at 24, 48, and 72 hours after transfection by Total RNA Extraction Kit (Parstous, Iran). The concentration of RNAs was measured at 260-280 nm in NanoDrop One. Easy cDNA Synthesis Kit (Parstous, Iran) was used to synthesize cDNA through reverse transcription reaction with stem-loop primers shown in Table 1 (17). SYBR Green Real-Time PCR Master kit (Parstous, Iran) was used for real-time PCR. The expression of hsa-piR-32877 was normalized to U6 expression. qRT-PCR was done using ABI Step One Plus (ABI, USA) system. The data calculation method was ΔΔCt. All of the processes

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discussed above were performed for CASP3 and CASP9 genes. The expression of CASP3 and CASP9 were analyzed to determine the apoptosis after hsa-piR-32877 suppression with antisense LNA GapmeRs. The expression of CASP3 and CASP9 was normalized to GAPDH expression. The sequence of primers used for real-time PCR showed in Table 1 (17, 21-23).

Table 1. Quantitative real-time PCR (qRT-PCR) primers.

primers	Sequences (5′ → 3′)	References
piR-32877Stem-loop	GTTGGCTCTGGTGCAGGGTCCGAGG TATTCGCACCAGAGCCAACCGAGTC	17
U6 Stem-loop	GTTGGCTCTGGTGCAGGGTCCGAGG TATTCGCACCAGAGCCAACTTTTAT	17
CASP3 Forward	TCCACAGCACCTGGTTATTATTC	21
CASP3 Reverse	ACTCAAATTCTGTTGCCACCTTTC	21
CASP9 Forward	ATTTGGTGATGTCGGTGCTC	23
CASP9 Reverse	TCACGGCAGAAGTTCACATTG	23
GAPDH Forward	TGCACCACCAACTGCTTAGC	22
GAPDH Reverse	GGCATGGACTGTGGTCATGAG	22
piR-32877 Forward	GTTTAGTTACTACTTTGATCGGTTT	17
U6 Forward	TTGCACGAGCGAAGCCGT	17
Universal Reverse (for piR-32877 and U6)	GTGCAGGGTCCGAGGT	17

Statistical analysis

In this study, all tests were performed three times. Data analysis was carried out using GraphPad Prism 9 (GraphPad, USA) software. Besides, graphs were drawn with GraphPad Prism 9. To analyze the differences between groups, the two-way ANOVA test was used. The data were shown as mean ± standard deviation. A $p < 0.05$ was considered to represent a statistically significant difference.

Results

Suppression of hsa-piR-32877 using antisense LNA GapmeRs

Since antisense LNA GapmeRs possess the FAM (6-Carboxyfluorescein) group, the efficiency of transfection was checked after 6 h of incubation by a Nikon Eclipse Ti-U fluorescence microscope. The efficiency of transfection was about 80% (Figure 1). After transfection, the expression of hsa-piR-32877 was investigated by qRT-PCR in control, antisense LNA GapmeR, and antisense LNA GapmeR negative control groups at 24, 48, and 72 hours. Results have shown that hsa-piR-32877 dramatically was down-regulated in antisense LNA GapmeR groups at 24, 48, and 72 hours in comparison with control and antisense LNA GapmeR negative control groups (***$p < 0.001$)(Figure 2).

Suppression of hsa-piR-32877 reduced cell proliferation

CFSE dye was used to analyze cell proliferation at 24, 48, and 72 hours after transfection, by the BD FACSCalibur flow cytometer. The cell proliferation decreased in the antisense LNA GapmeR negative control groups compared with control groups but was not significant, due to the transfection reagent toxicity, cell viability minimally decreased in LNA GapmeRs negative transfected cells compared to the normal controls but this difference was not at all statistically significant. Although the cell proliferation in antisense LNA GapmeR groups decreased after 72 hours in the cell line sample, it is not substantial in comparison with control and antisense LNA GapmeR negative control groups (Figure 3).

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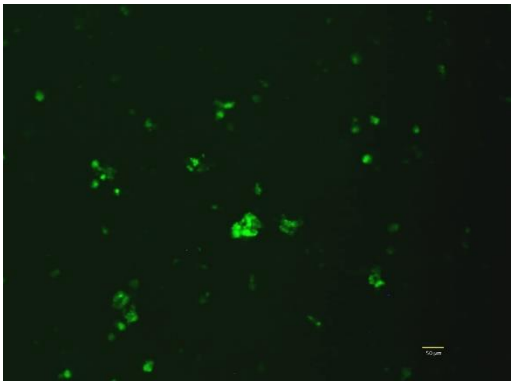

Fig.1. AML cells was transfected by antisense LNA GapmeRs and the efficiency of transfection was analyzed with the fluorescent microscope. Images show that most of the cells are transfected by antisense LNA GapmeRs. Scale bar: 50 µm.

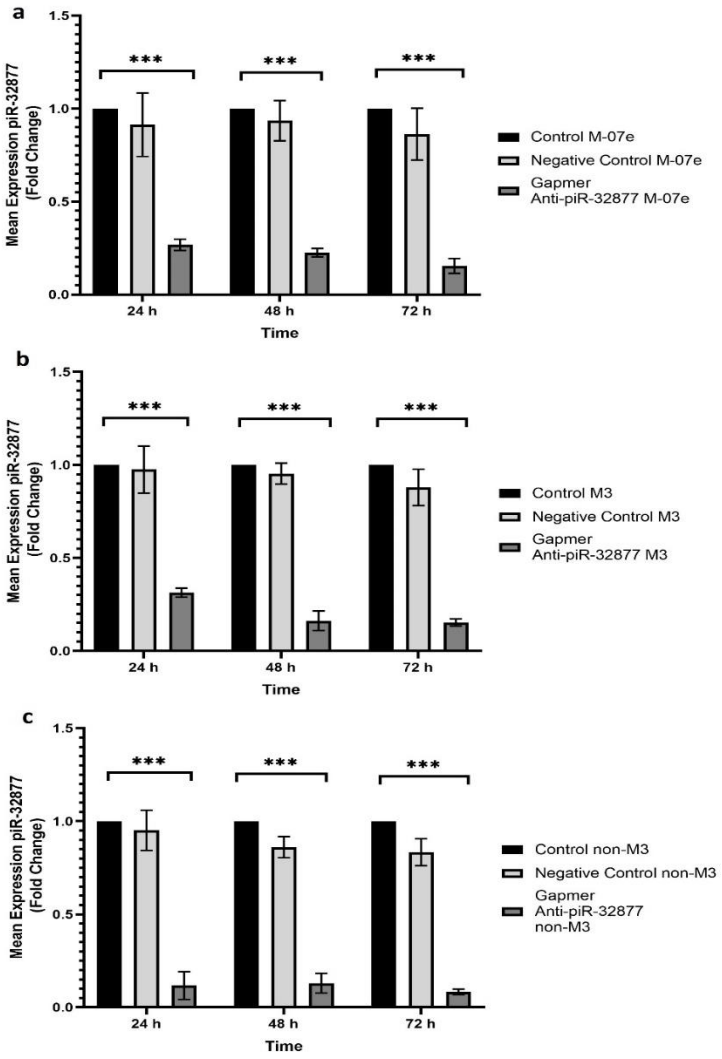

Fig.2. After transfection, the expression of piR-32877 was analyzed by qRT-PCR at 24, 48, and 72 hours. Control groups were not transfected, so they were considered reference groups for comparison with other groups. Data analysis was performed using ΔΔCt method. The data is shown in the graph as mean ± SD of triple independent experiments (***$p < 0.001$). a. M-07e b. M3 patient c. non-M3 patient.

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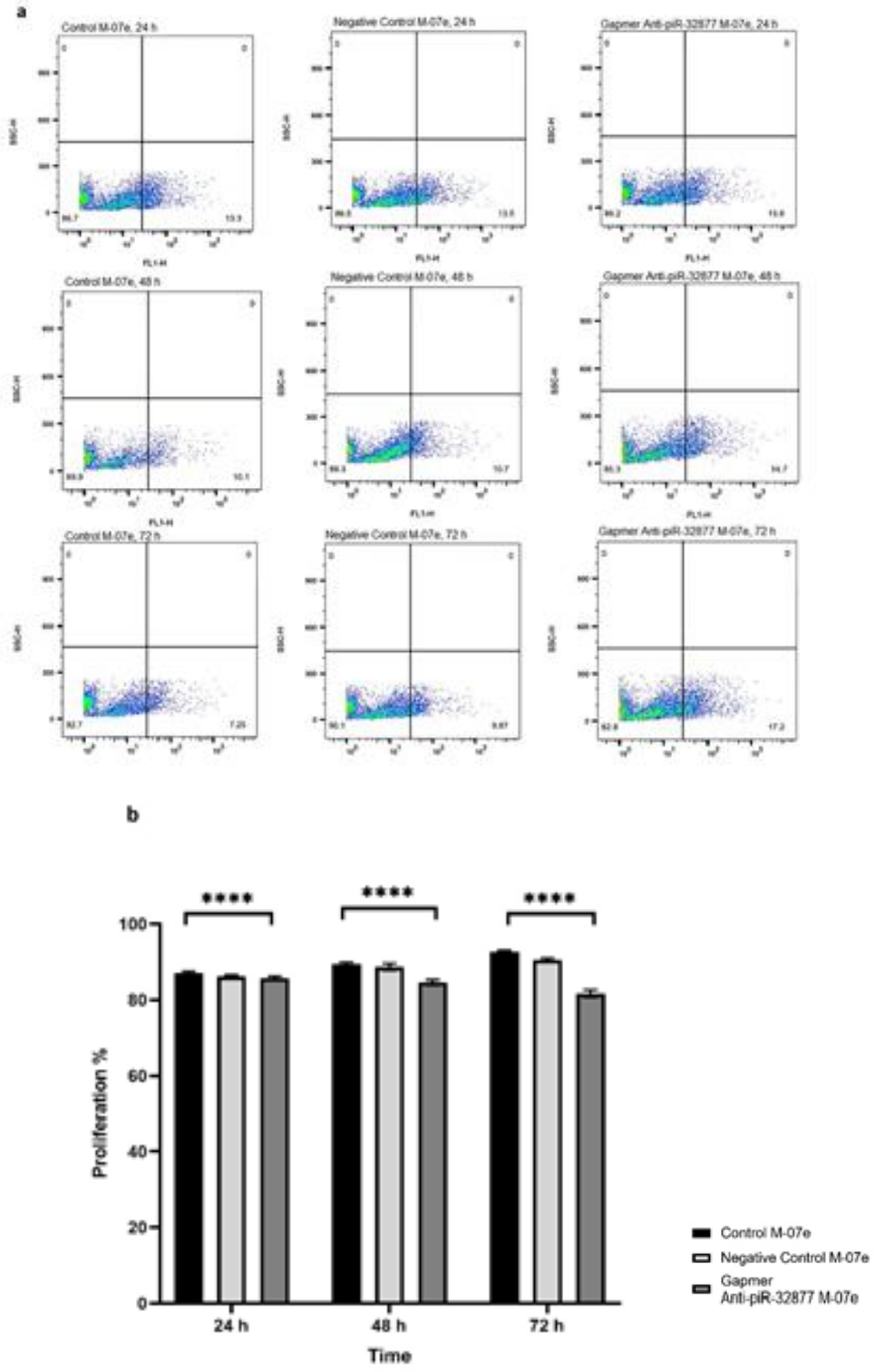

Fig.3. Assessment of cell proliferation by CFSE kit at 24, 48, and 72 hours in the M-07e cell line after transfection. a. Flow cytometry was performed using 492-nm excitation and 517-nm emission wavelengths. b. The data is shown in the graph as mean ± SD of triple independent experiments (****$p < 0.0001$).

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The cell proliferation decreased in antisense LNA GapmeR groups in both patient samples at 24, 48, and 72 hours. As the results showed, the cell proliferation in antisense LNA GapmeR groups belonging to patient samples decreased by about 50% after 72 hours (****p < 0.0001) (Figures 4-5).

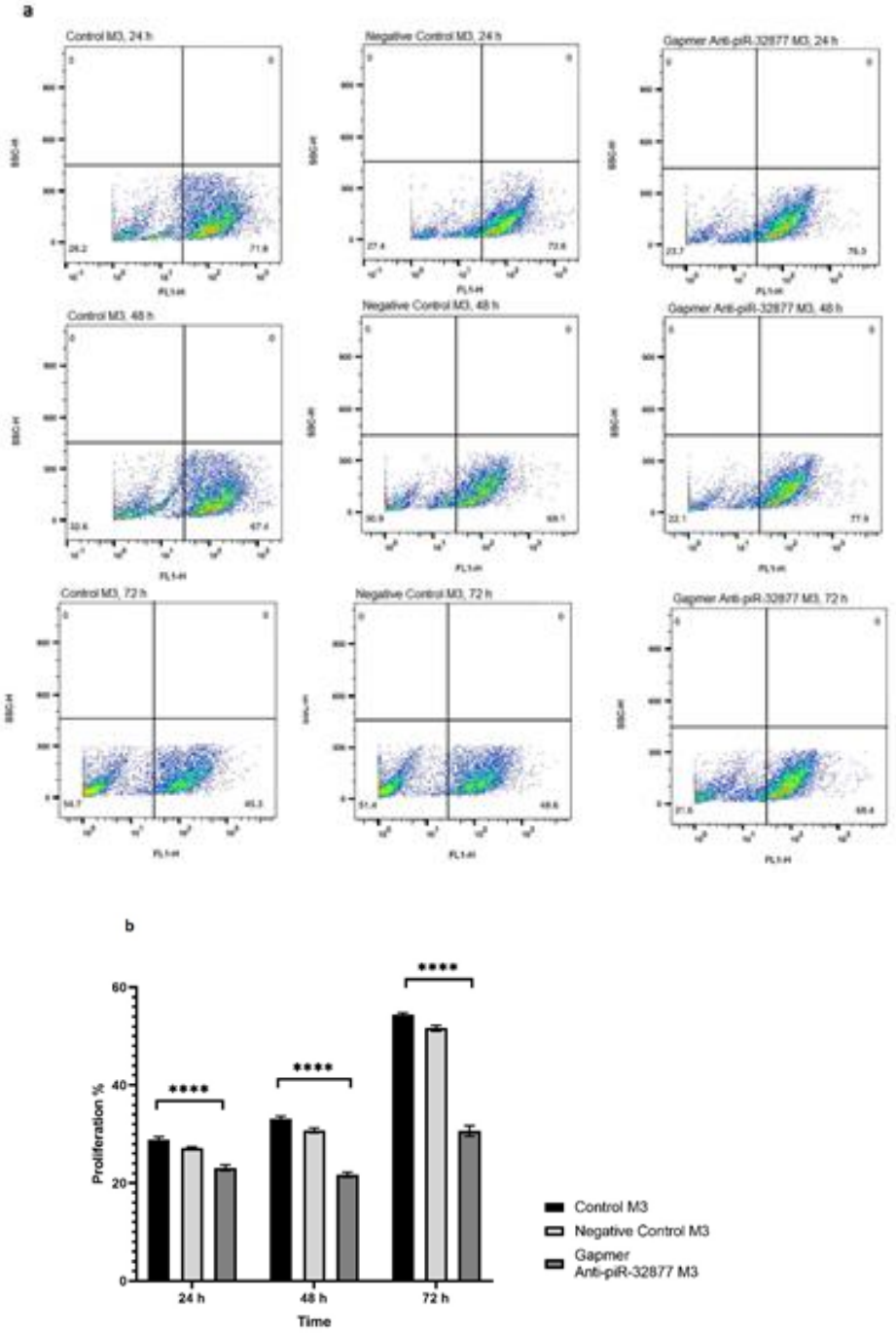

Fig.4. Assessment of cell proliferation by CFSE kit at 24, 48, and 72 hours in the M3 patient after transfection. a. Flow cytometry was performed using 492-nm excitation and 517-nm emission wavelengths. b. The data is shown in the graph as mean ± SD of triple independent experiments (****$p < 0.0001$).

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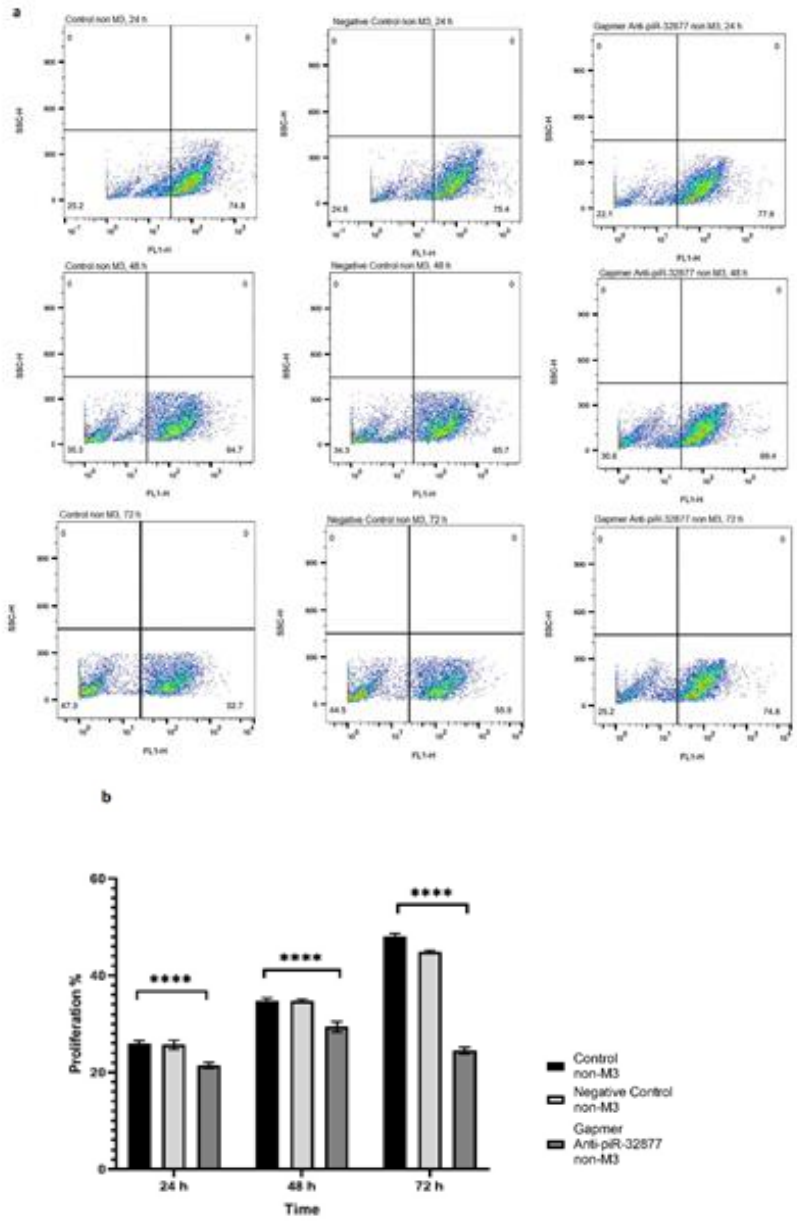

Fig.5. Assessment of cell proliferation by CFSE kit at 24, 48, and 72 hours in the non-M3 patient after transfection. a. Flow cytometry was performed using 492-nm excitation and 517-nm emission wavelengths. b. The data is shown in the graph as mean ± SD of triple independent experiments (****p < 0.0001).

Suppression of hsa-piR-32877 increased apoptosis

To evaluate apoptosis following hsa-piR-32877 suppression after transfection, the expression of CASP3 and CASP9 was measured at 24, 48, and 72 hours for all of the groups by qRT-PCR. Results have shown that both CASP3 and CASP9 increased in antisense LNA GapmeRs groups compared with control and antisense LNA GapmeR negative controls groups in both the cell line and patient samples. Besides, the highest amount of apoptosis was observed at 48 and 72 hours in antisense LNA GapmeR groups for both the cell line and patient samples (***p < 0.001) (Figure 6).

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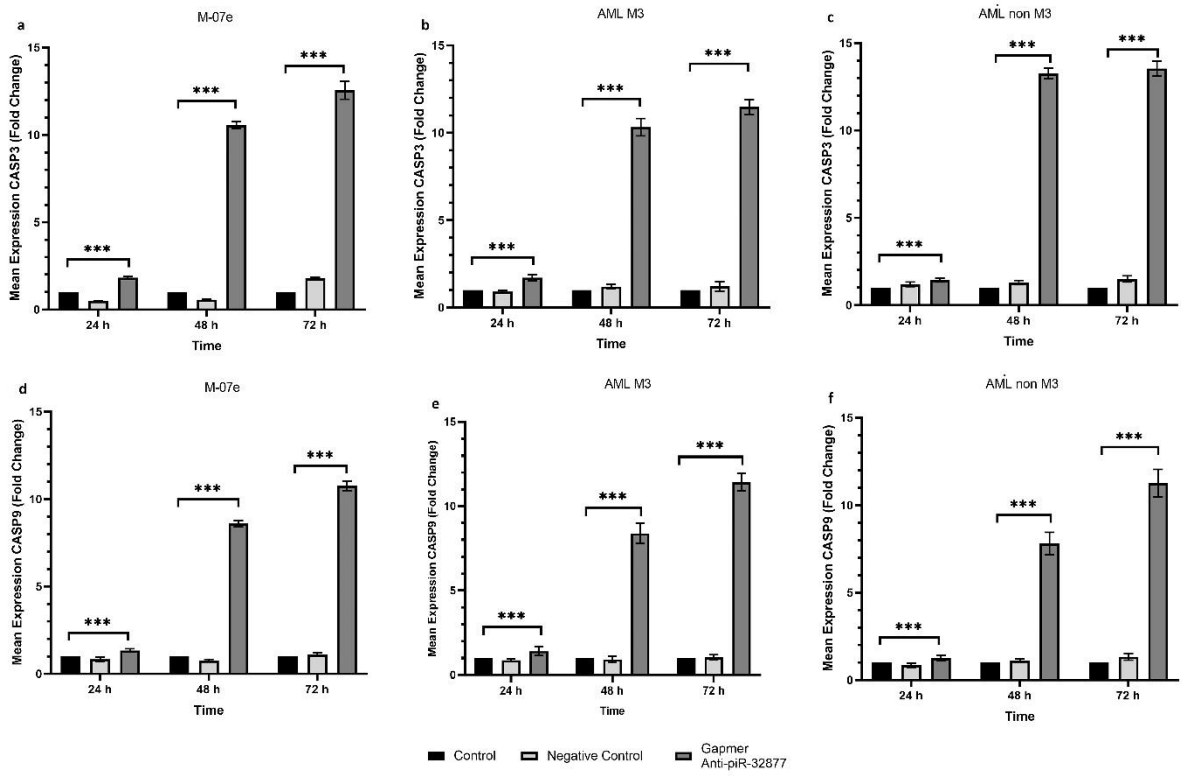

Fig.6. To analyze apoptosis, the expression of *CASP3* and *CASP9* were evaluated by qRT-PCR at 24, 48, and 72 hours after transfection. Data analysis was performed using ΔΔCt method. The data is shown in the graph as mean ± SD of triple independent experiments (***$p < 0.001$). *CASP3* expression level in M-07e (a), M3 patient (b), and non-M3 patient (c); *CASP9* expression level in M-07e (d), M3 patient (e), and non-M3 patient (f).

Discussion

Acute myeloid leukemia (AML) is a complex and heterogeneous hematological disorder due to the uncontrolled proliferation of myeloid progenitor cells. Results showed that more than 20 000 AML cases per year were diagnosed in the United States. Despite treatment strategies, most patients die, result of resistance to treatments (24, 25). So, due to the prevalence of AML and relapse of this disease, novel treatment strategies are needed.

Studies have shown that piRNAs are associated with different cancers, and act as oncogene or tumor suppressors. Besides, overexpression of piRNAs and their effects on leukemia have been observed (26, 27). Yan H *et al.* found that piRNA-823 acts as an oncogene molecule and promotes multiple myeloma through DNA methylation and angiogenesis regulation (28).

Cell proliferation and apoptosis are important processes in the cells. So, loss of balance between cell proliferation and apoptosis could be due to cancer such as AML (29, 30). Caspase 9 and caspase 3 play vital roles in the apoptotic pathway. Caspase 9 promoted ROS (Reactive oxygen species) production through blockage of the accessibility of cytochrome c to complex III in mitochondria. Besides, caspase 3 is activated by caspase 9 and results in the prevention of ROS production (32). Studies have shown that piRNAs

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expression affects cell proliferation and apoptosis through the regulation of IPK3/AKT/mTOR and CDK4/6 pathways (32).

Ghaseminezhad Z *et al*. have studied the expression profile of piRNAs in AML via the GEO database. Authors have reported that piR-32877 and piR-33195 were overexpressed in this disease (17). In the present study, we have suppressed piR-32877 in both AML patients and the cell line by antisense LNA GapmeRs. The piR-32877 substantially decreased after antisense LNA GapmeRs transfection which was approved by qRT-PCR. The CFSE assay displayed that the cell proliferation was inhibited after the suppression of piR-32877. Since caspase 3 and caspase 9 carried out important roles in apoptosis, we studied the expression of these caspases after piR-32877 suppression. Results have shown that CASP3 and CASP9 were significantly up-regulated after transfection at 24, 48, and 72 hours in both cell line and patient samples that were confirmed by qRT-PCR. Hence, we may be able to induce apoptosis by the blockage of piR-32877 in AML. In total, the present study has shown that degradation of piR-32877 after transfection of the cells by antisense LNA GapmeRs; remarkably decreased the cell proliferation and induced apoptosis by enhancing the expression of CASP3 and CASP9.

The regulation of apoptosis and increasing cell proliferation by piR-32877 inhibition could be a novel therapeutic method in combination with other treatments for AML.

Taken together, our data showed that inhibition of piR-32877 by antisense LNA GapmeRs decreased cell proliferation and induced apoptosis in the AML cell line and patient samples. Besides, piR-32877 suppression results in increased expression of CASP3 and CASP9 which play important roles in apoptosis. Therefore, degradation of piR-32877 may be an efficient strategy for controlling blast cell proliferation and apoptosis induction which could be considered a novel treatment for AML. More studies are required to assure the role of piR-32877 in AML diagnosis.

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