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RESEARCH ARTICLE

Reduce proliferation of human bone marrow cells from acute myeloblastic leukemia with minimally differentiation by blocking lncRNA PVT1

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Abstract

Purpose Acute myeloblastic leukemia with minimally differentiation (AML-M0) is a subtype of acute leukemia with poor prognosis. The recent studies have shown that long non-coding RNAs (lncRNAs) play an important role in different cellular processes, such as cell cycle control and proliferation. Plasmacytoma variant translocation 1 (*PVT1*) is one of those lncRNAs that is significantly upregulated in AML. LncRNAs could be downregulated or blocked by locked nucleic acids (LNA) which are oligonucleotide strands.

Methods In this study, lncRNA *PVT1* was blocked by antisense LNA GapmeRs in human bone marrow cancerous blast cells. Cells were transfected with *PVT1* antisense LNA GapmeRs at 24, 48, and 72 h post-transfection. Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was accomplished to evaluate the *PVT1* and *c-Myc* expression. Cell viability was evaluated by MTT assay, and apoptosis and necrosis were assessed by Annexin V/propidium iodide staining assay.

Results The results of this study indicated that the downregulation of *PVT1* in blast cells could induce apoptosis, and necrosis and reduce cell viability. The expression of *c-Myc* was downregulated by blockage of *PVT1* and it shows that the expression of these two genes are correlated.

Conclusion The findings declare that inhibition of *PVT1* could be a new target in the treatment of AML-M0 and help to approach more to treatments with fewer side effects.

Keywords Acute myeloblastic leukemia · *PVT1* · *c-Myc* · LNA GapmeRs

Introduction

In the past several years, there has been huge progress in molecular studies by knowing non-coding RNAs (ncRNAs) also known as non-messenger RNA (nmRNA) or functional RNA (fRNA) and their function [1]. ncRNAs thought to be the noise of the transcription system [2]. These non-coding RNAs consist of microRNAs (miRNAs), small nuclear RNA (snRNA), PIWI-interacting RNA (piRNAs), and long non-coding RNA (lncRNAs) [1]. Long non-coding RNAs are which longer than 200 nucleotides in length and like messenger RNAs (mRNA) are transcribed by RNA pol II. lncRNAs get spliced and are capped and usually polyadenylated like mRNAs [1, 3, 4]. lncRNAs can be divided into four groups based on their loci. These four groups are sense, antisense, intronic, and intergenic [5]. They play role in important cellular processes, such as chromosome structure modulation, transcription, splicing, microRNAs sequestration, imprinting, epigenetic regulation, cell cycle

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control, nuclear and cytoplasmic trafficking, and translational and post-translational modifications [5–7]. Owing to the involvement of lncRNAs in these crucial processes, they can have associations with some fatal complex diseases, such as diabetes, cardiovascular disease, and cancer [3, 6]. Based on the World Health Organization (W.H.O) report, it has been approximated that death due to cancer is going to be increased from 80 to 100% in the Middle East in the next decade. Leukemia is one of the most common cancers [8]. Acute myeloblastic leukemia (AML) is a type of leukemia that is heterogeneous that is characterized by the uncontrolled proliferation of immature myeloblastic cells [9]. This proliferation of immature progenitors (blast cells) leads to severe infections, anemia, and hemorrhage. One diagnosis of AML is the identification of more than 20% of blast cells in bone marrow or peripheral blood [10]. The French–American–British (FAB) classification divided AML into eight subtypes (M0 through M7) based on the cytochemical staining, morphological characteristics of the cancerous cells [11]. AML-M0 define as minimally differentiated acute myeloblastic leukemia. Blast cells in AML-M0 are large and have a heterogeneous morphological feature, and they have agranular cytoplasm, but do not have Auer rods [12].

It has been noted that dysregulation of lncRNAs can be associated with different types of cancer like breast, prostate, colorectal, and leukemia and they can act as tumor suppressors or oncogenes [13, 14]. One of the lncRNAs that have been taken into consideration is the plasmacytoma variant translocation 1 (*PVT1*) which is a long intergenic noncoding RNA and the locus of the *PVT1* gene in human, 8q24.21, is about 30 kb and is 53 kb downstream of *MYC* gene [3, 15]. It has been reported that the expression and function of this lncRNA are associated with different cancers, including breast, bladder, pancreas, and leukemia [9, 16–18]. It has been declared that *PVT1* can have a role in cell proliferation, angiogenesis, and apoptosis escape, encoding miRNAs like miR-1204 and miR-1207-5p, also in DNA rearrangement and interacting with *MYC* [15, 19].

Amplification or rearrangement in the *PVT1* gene location has been established; furthermore, Liu et al. mentioned that the amplification in this gene has been detected in 10% of AML patients [5, 9]. Through the amplification process, *PVT1* can generate new fusion genes that have been proved to play a role in tumor development, it also can produce fusion gene by translocation. Although *PVT1* is a non-protein coding gene but with fusion to other genes, it can produce chimer protein like *PVT1–AKT3* which is a shorter protein. *PVT1* can also fuse to its neighbor gene, *MYC*. The fusion of *PVT1–MYC* has happened in many cancer cases with a high copy number of region 8q24 [15].

There is a positive feedback between *MYC* and *PVT1* and it has been demonstrated that they involved in the same pathways. *c-Myc* itself, as a transcription factor, can increase *PVT1* transcription by binding into tow enhancer E-boxes that are in the promoter of the *PVT1* gene. Accordingly, *c-Myc* increases the transcription of *PVT1* and *PVT1* protects *c-Myc* from degradation. lncRNA *PVT1* can stabilize *c-Myc* by blocking the Thr58 witch is a phosphorylation site and when it is phosphorylated *c-Myc* can be degraded by the ubiquitin pathway [15, 20].

According to the function of *PVT1*, for clarifying its potential for being a therapeutic target, a novel therapeutic agent is needed.

Locked nucleic acids (LNA) are DNA oligonucleotide strands that have locked furanose structure due to the 2′-O,4′-C-methylene linkage and show resistance across exonucleases, in addition, they have an affinity to the complementary DNA or RNA strand. LNA-GapmeRs are DNA oligonucleotide strands that are locked by LNAs at the 5′ and 3′ end and it can create a heteroduplex with RNA strand that can leads to degradation of the RNA strand by RNase-H [21, 22].

Materials and methods

Bone marrow samples were taken from patients with informed consent in the Sayed-al Shohada Hospital (Isfahan, Iran). The subtype of AML was qualified by a pathologist in the hospital laboratory based on the FAB classification of AML.

Cell culture

Blast cells were separated from bone marrow by using Ficoll. Five ml phosphate-buffered saline (PBS) was added genteelly into a 5 ml bone marrow sample. About 8 ml of this mixture was added slowly into 3 ml of Ficoll and was centrifuged for 20 min at 3000 RPM. The mononuclear cells were separated, then an equal volume of PBS was added into the cells then centrifuged at 2500 RPM for 5 min. The cell's pellet was dissolved with cell culture medium which consists of RPMI 1640 that was supplemented with 15% of fetal bovine serum (FBS), 1% of Pen–Strep, 4 microL of granulocyte-colony stimulating factor (G-CSF) per 100 ml of the culture medium, then cells were cultured at 37 °C in a humidified incubator with 5% CO_2 in 25-cm^2 culture flasks, and the cells were passaged two or three times a week.

Cell transfection

lncRNA *PVT1* sequence was obtained from a reputable site: https://www.lncrnadb.org (accession id: NR_003367.2).

Antisense LNA GapmeRs and antisense LNA GapmeR Negative controls oligonucleotides (ALGNC) for lncRNA

PVT1 were purchased from the Qiagen. Antisense LNA GapmeRs and ALGNC were labeled at their 5′-end with a fluorescent dye, 6-FAM (6-carboxyfluorescein). Cell transfection was performed using the PolyFect™ transfection reagent kit (Qiagen) according to the manufacturer's instructions. When cells were in exponential phase, they were seeded into six-well culture plates containing 1.8 ml RPMI 1640 per well without antibiotics and FBS.

Nine microliters of antisense LNA GapmeRs was dissolved in 300 microL of RPMI, 74 microL of transfection reagent was dissolved in 900 microL of RPMI, and 300 microL of dissolved transfection reagent was added into antisense LNA GapmeR and incubated at room temperature in the dark for 10 min then 200 microL of this mixture was added into GapmeR group well, and the plate was rotated cautiously while adding the mixture to ensure the distribution over the plate. The same procedure was done for the ALGNC group as well. In addition, for the mock group, 200 microL of dissolved transfection reagent was added into each mock group's well. After 6 h of incubation, FBS and antibiotics were added to the cells, and then the cells were incubated for 24, 48, and 72 h. Untreated cells, the mock group cells, and cells transfected with ALGNC were cultured in parallel with antisense LNA GapmeRs transfected cells.

Transfected cells with antisense LNA GapmeRs were seen by a fluorescence microscope due to the fluorescent dye.

Real-time PCR

lncRNA real-time PCR was performed to determine the efficiency of lncRNA *PVT1* inhibition by antisense LNA GapmeRs. Briefly, total RNA was extracted with RNA isolation kit (Favorgen, Taiwan) at 24, 48, and 72 h after transfection. RNA concentration and purity were measured at an optical density (OD) of 260–280 nm with spectrophotometer. Then, the total RNA which was isolated was reverse transcribed into complementary DNA (cDNA) using the Universal cDNA Synthesis Kit (Qiagen). Real-time PCR was performed using the SYBR Green master mix kit (Qiagen) with the following primers for *PVT1*: forward: 5′GCACTCTGG ACGGACTTGAG3′, reverse: 5′TGTCCACTAGCAGCA ACAGG3′; and *c-Myc* primers: forward: 5′CATACATCC TGTCCGTCCAA3′ reverse: 5′GAGTTCCGTAGCTGTTCA AGT3′. *PVT1* and *c-Myc* expression level in each sample were normalized to the GAPDH expression level, with the following primers, forward: 5′GGTGTGAACCATGAGAAG TATGA3′, reverse 5′GAGTCCTTCCACGATACCAAAG3′.

The ABI Step One Plus (ABI, USA) instrument was used for real-time PCR experiments and the ΔΔCt method was used for data calculation.

All assays were performed in triplicate, and the specificity of each PCR was confirmed with LinregPCR software version 2017.1.0.0.

MTT assay

10 microL of 3-[4, 5-dimethylthiazole-5-yl]-2,5-diphenyltetrazolium bromide (MTT) was added into 100 microL of transfected cells in 96 well plates and after 3 h, 100 microL of dimethyl sulfoxide (DMSO) was added into cells. The absorbance was measured at OD = 590 nm by ELISA reader.

Apoptosis and necrosis

The FITC Annexin V Apoptosis Detection Kit with propidium iodide (PI; BioLegend, San Diego, CA) was used for the detection of apoptosis and necrosis of the cells.

500 microL of PBS was added into cell pellet, and then centrifuged for 10 min at 2000 RPM after which 100 microL of the prepared buffer (based on kit instruction) was added to each tube. 3 microL of annexin-V binding buffer solution and 5 microL of PI staining were added to each tube except the control group and incubated at room temperature in the dark for 15 min. After incubation, another 400 microL of the prepared buffer was added into the tubes.

Then, the tubes were moderately vortexed and immediately analyzed using an FACSCalibur flow cytometer (Becton Dickinson), equipped with CellQuest software (Becton Dickinson) [23].

Statistical analysis

All tests were performed in triplicate, and the results were analyzed with SPSS version 25 software. Also, Graphpad prism version 8.3.0.538 was used to draw graphs. The difference between the groups was evaluated, using the two-way ANOVA test. The data are expressed as mean ± standard deviation. For all statistical analyses, $p < 0.05$ was considered statistically significant.

Results

Degradation of lncRNA *PVT1* by antisense LNA GapmeRs and q-RT PCR

After the transfection of antisense LNA GapmeRs into the cells, the transfection efficiency was determined by fluorescence microscopy due to the FAM group in the oligonucleotide strands. The transfection level was determined by more than 90% by flow cytometry (Fig. 1). q-RT PCR was performed to measure the expression of *PVT1* in four different

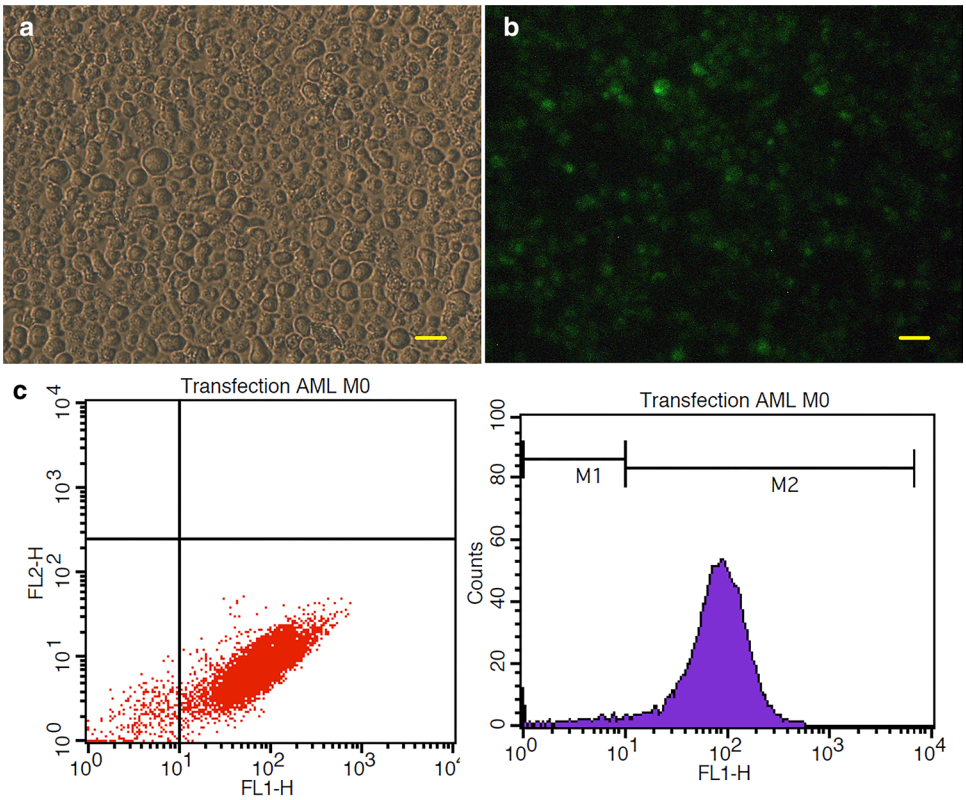

Fig. 1 AML-M0 blast cells were transfected with 6-FAM labeled antisense LNA GapmeRs and then the transfection efficiency was evaluated by a fluorescent microscope and flow cytometer. Phase contrast (**a**), fluorescent (**b**) images of the same field of blast cells display that the majority of cells were transfected. flow cytometer (**c**) also confirmed that a large number of cells were transfected. Scale bars: 50 µm.

cell groups (control, mock group, ALGNC, Antisense LNA GapmeRs) 24, 48, and 72 h after transfection. *PVT1* expression was decreased significantly in the antisense LNA GapmeRs group at all of the three-time points compared to other groups ($p < 0.001$) (Fig. 2).

Degradation of lncRNA *PVT1* reduced cell viability

To evaluate the effect of *PVT1* inhibition on cell viability, the MTT test was performed at 24, 48, and 72 h after transfection. Compared to the control group, the viability in all other groups was reduced, but the reduction in antisense LNA GapmeRs group was notably significant (Fig. 3). The cell viability reduction in this group was about 50% in 72 h after transfection. The reduction in other groups may have been due to the toxicity of transfection reagents or the senescence of the cells.

Degradation of lncRNA *PVT1* increased apoptosis and necrosis

To determine the effect of *PVT1* inhibition on apoptosis and necrosis Annexin-V/PI double staining was carried out after transfection at 24, 48, and 72 h. Due to the transfection reagent toxicity, the apoptotic ratio was high in all groups, but it was more significant in the antisense LNA group ($p < 0.001$). The highest amount of apoptosis was in the LNA

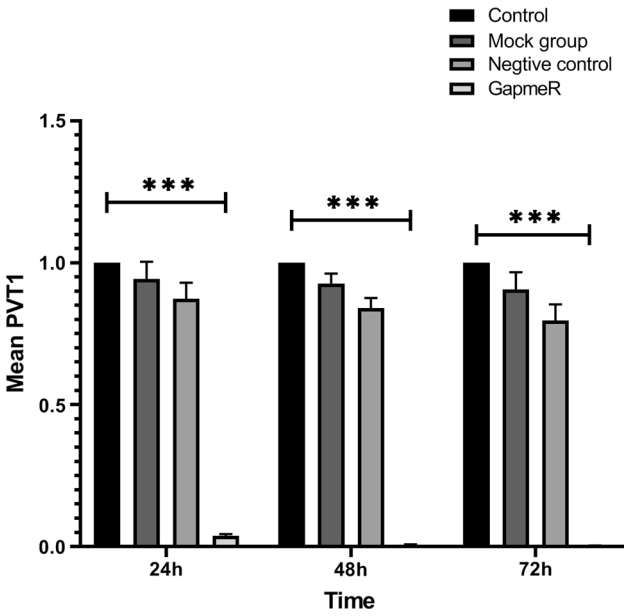

Fig. 2 The *PVT1* expression level was determined, using qRT-PCR assay at 24, 48, and 72 h after transfection. The ΔΔCt method was applied for data analysis, and the untreated cells were used, as a control group and as a reference for comparison with other groups. The data are presented as mean ± SD of three independent experiments (***$p < 0.001$)

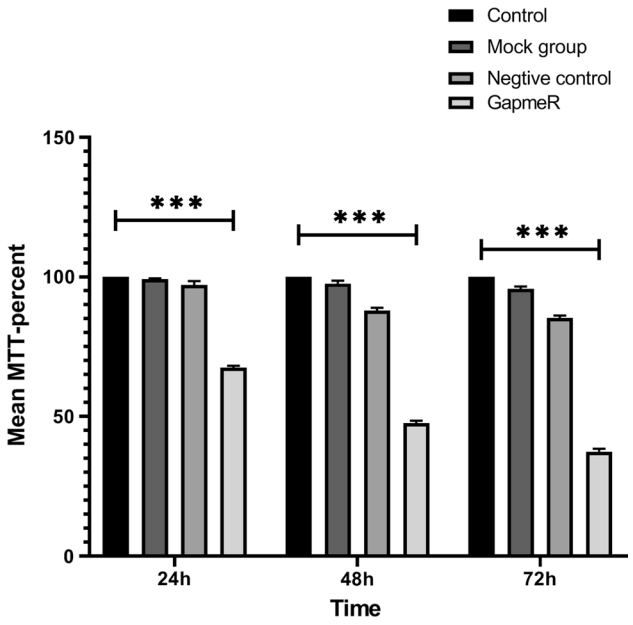

Fig. 3 Blast cells' viability was measured by MTT assay at 24, 48, and 72 h after transfection. The viability of the untreated cells at each time point was considered as 100%, and the viability of other groups is displayed as the percentage of the untreated cells at the same time point. The data are presented as the mean SD of three independent experiments (***$p < 0.001$)

GapmeRs group compared to other groups. Generally, the results reveal that *PVT1* degradation induced apoptosis in the cells. (Fig. 4). Simultaneously, necrosis was measured as well. The necrosis ratio was significantly higher in the LNA GapmeRs group at all three-time points, which explains why apoptosis rate was lower in the LNA GapmeRs group compared to other time points results, the cells were entered in necrosis phase. This result represents that *PVT1* blockage by antisense LNA GapmeRs may have induced necrosis in human cancerous blast cells (Fig. 5).

Degradation of lncRNA *PVT1* influenced the *c-Myc* expression

qRT-PCR was performed for c-Myc as well as PVT1 in control, mock group, ALGNC, and antisense LNA GapmeRs group at three-time points after transfection. The expression of c-Myc was lower in the LNA GapmeRs group as compared to others. In this group, c-Myc expression was almost the same amount at 24 and 72 h and it was a little more in 48 h post-transfection. It can be concluded that the expression of *PVT1* can influence *c-Myc* expression (Fig. 6).

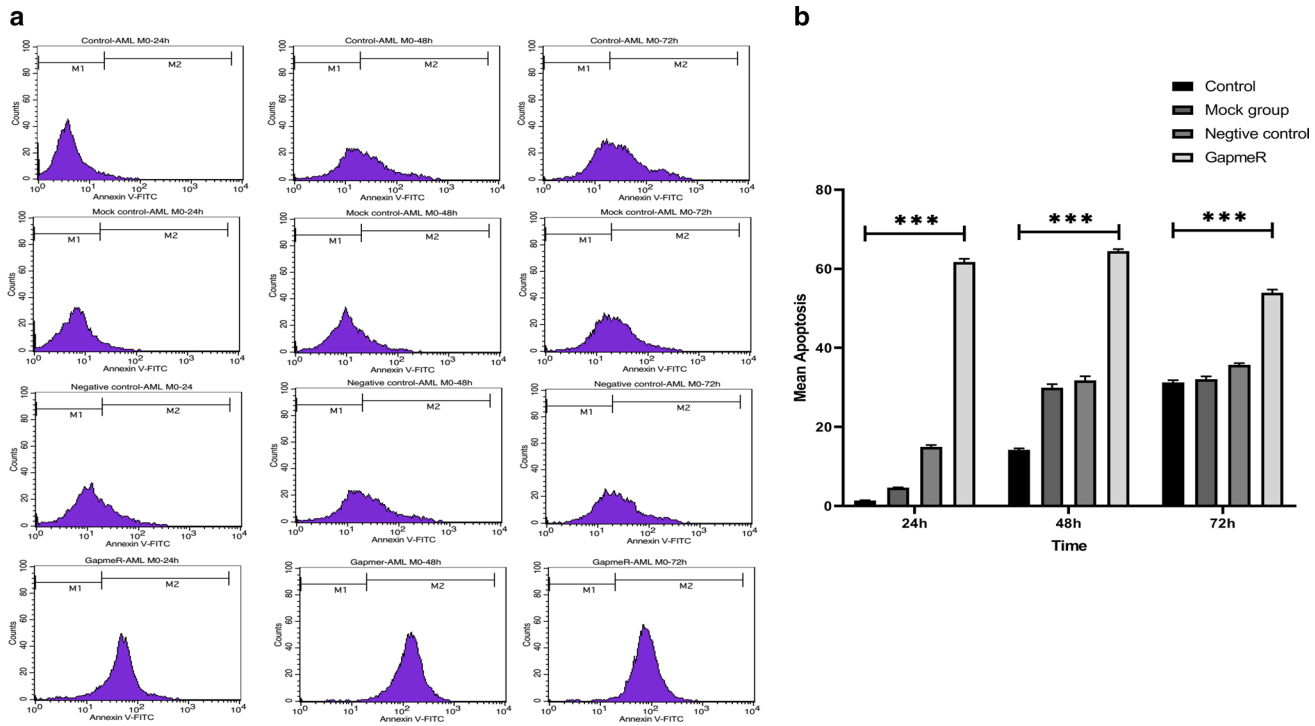

Fig. 4 Blast cells apoptosis were evaluated by Annexin V staining at 24, 48, and 72 h after transfection. Flow cytometry analysis was performed, using 488-nm excitation and a 518-nm bandpass filter for the fluorescein detection. Representative cytofluorometric graphs are shown (**a**). The apoptosis of the untreated cells in each time point was considered as-0%, and the apoptosis of other groups is presented as the percentage of the untreated cells at the same time point. The ratio of apoptotic cells was increased by *PVT1* antisense LNA GapmeRs at three-time points compared to other cell groups. The data shown in the graph are presented as mean ± SD of three independent experiments (***$p < 0.001$) (**b**)

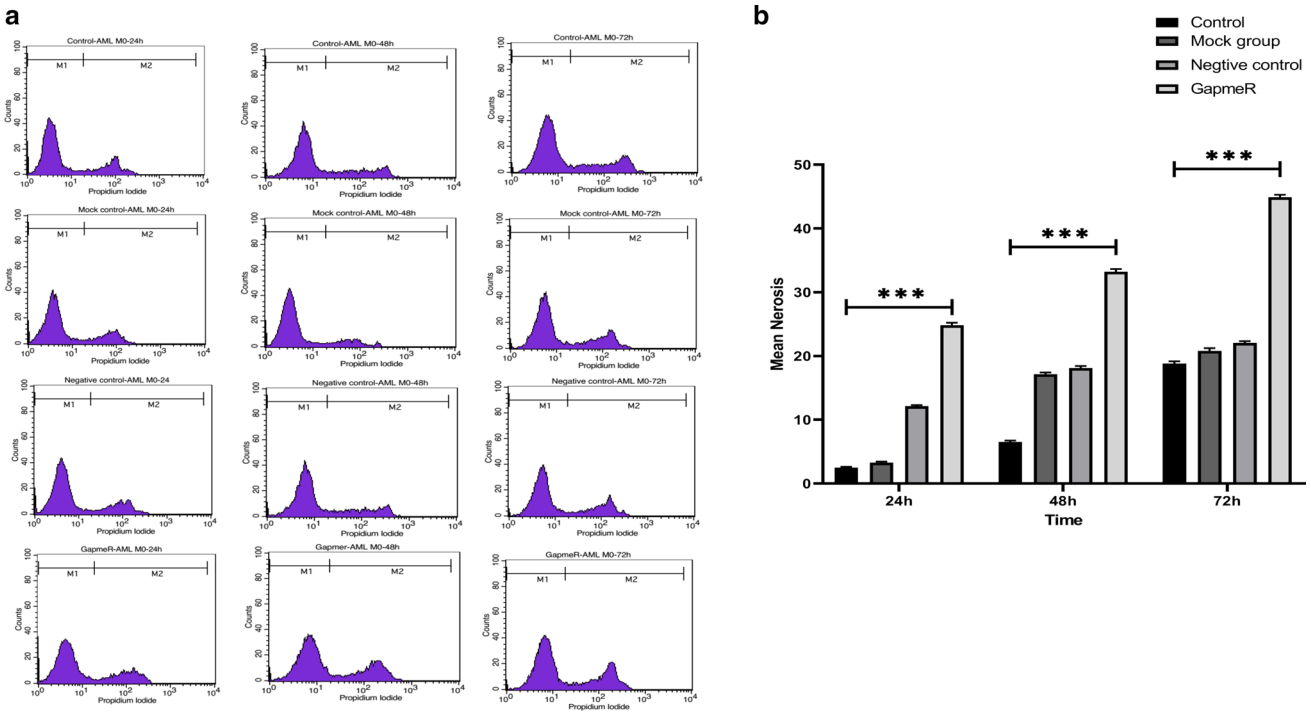

Fig. 5 Blast cells late apoptosis/necrosis were evaluated by PI staining at 24, 48, and 72 h after transfection. Flow cytometry analysis was performed using a filter of 617 nm for PI detection. Representative cytofluorometric graphs are shown (**a**). The necrosis of the untreated cells in each time point was considered as ~ 0% and the necrosis of other groups is presented as the percentage of untreated cells at the same time point. The ratio of necrotic cells was increased by *PVT1* antisense LNA GapmeRs at three-time points compare to other cell groups. The data shown in the graph are presented as the mean ± SD of three independent tests (***$p < 0.001$) (**b**)

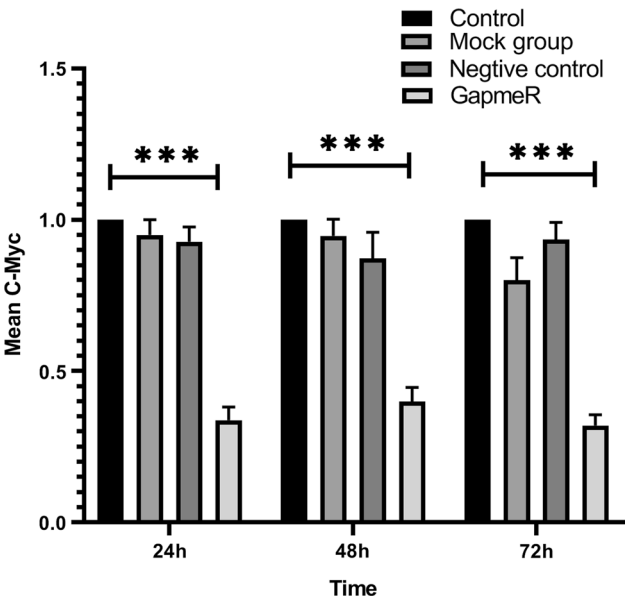

Fig. 6 The *c-Myc* expression level was determined, using qRT-PCR assay at 24, 48, and 72 h after transfection. The ΔΔCt method was used for data analysis, and the untreated cells were considered as a control group and as a reference for comparison with other groups. The data are presented as mean ± SD of three independent experiments (***$p < 0.001$)

Discussion

Abnormal proliferation of myeloblastic cells can cause AML, which is a heterogeneous hematological malignancy. Although the treatments of leukemia are widespread nowadays, most patients still relapse [6]. Epidemiologically, the incidence of AML has been increasing however; the death rate has been decreased through the decades. Notably, this reduction is not due to anti-leukemic drugs, it is because of antimicrobials, better blood bank services, more patient care and allogeneic stem cell transplants. Despite all these signs of progress, the backbone of therapy remains a combination of cytarabine- and anthracycline-based regimens, which has several side effects for the patient [7]. The recent detailed data about AML molecular ontogeny and molecular pathways have incited new-targeted therapies for this cancer [24, 25]. LncRNAs are one of the regulators of cell proliferation and differentiation, and it has been declared that lncRNA expression followed a lineage-specific pattern during early hematopoiesis, which is an important factor in causing leukemia, including AML. They are correlated with some famous protein-coding genes in the regulation of hematopoiesis [26]. Delás et al. discovered that leukemia blasts show a myeloblastic differentiation phenotype when some of these lncRNAs were depleted and their data indicate

that this effect is mediated via effects on the MYC oncogene. LncRNAs and coding genes show the same expression patterns across hematopoietic differentiation due to this study. Three lncRNAs appeared to be passable candidates required for leukemia progression, as their depletion led to leukemic blast differentiation; one of these lncRNAs was *PVT1* [27]. The role and expression level of this lncRNA has been studied in different types of cancer. Bioinformatics analysis showed that *PVT1* regulates VEGFC expression through miR-128 as a competing endogenous RNA (ceRNA) in bladder cancer [11]. Yang et al. found that *PVT1* was notably high and associated with aggressive progression and poor prognosis in non-small cell lung cancer [28]. It has been reported that the lncRNA *PVT1* promotes KLF5/beta-catenin signaling to drive triple-negative breast cancer tumorigenesis [12]. lncRNA *PVT1* was announced to be overexpressed in colorectal cancer tissues as compared to paired-adjacent normal tissues. The high expression of lncRNA *PVT1* predicts a later tumor stage and poorer tissue differentiation [29]. Kong et al. investigated the expression and role of *PVT1* in gastric cancer and found that the higher expression of *PVT1* was significantly correlated with deeper invasion depth and advanced tumor, node, and metastasis (TNM) stage. Multivariate analyses revealed that *PVT1* expression served as an independent predictor for overall survival [30].

In the present study, antisense LNA GapmeRs were used to knockdown lncRNA *PVT1* in AML-M0 blast cells, which were separated from the patient bone marrow sample. It was confirmed by real-time qPCR that by using LNA GapmeRs, *PVT1* expression was downregulated. It was clarified by MTT assay that the downregulation of *PVT1* is associated with a reduction in cell viability. In the following, apoptosis and necrosis were performed, and was confirmed that the apoptosis/necrosis rate was higher in the group that LNA antisense GapmeRs were used to knockdown *PVT1*.

The relationship between *PVT1* and *c-Myc* has been proven in many studies. C-Myc increases the transcription of *PVT1* by binding to E-boxes located in the *PVT1* promoter region, which results in the increased expression of *PVT1*. *PVT1* then prevents the degradation of *c-Myc* and maintains a high *c-Myc* protein level. Although it was claimed that the expression level of *PVT1* is correlated with the C-MYC protein level and its stability, not its expression level [13], in this study, the C-MYC expression level was also determined as *PVT1*. Real-time q-PCR revealed that *c-Myc* expression level was much lower in the group that was treated with antisense LNA GapmeRs against *PVT1*.

In most human cancers, the *c-Myc* expression is deregulated and/or significantly increased, if *c-Myc* is directly inhibited, therapeutic interventions will have a strong impact on patients [13], therefore, it is needed to target another molecule, which is more harmless for patients. LncRNA *PVT1* is a good candidate to be targeted with small molecules. In the present study, antisense LNA GapmeRs, which are stable oligonucleotide strands, were used against lncRNA *PVT1* for its degradation and caused apoptosis and necrosis and effect on *c-Myc* expression as well.

PVT1 inhibition along with other treatments for cancers, including AML, could be a promising targeted therapy with lower side effects. In this study, bone marrow sample of one patient was used to examine whether lncRNA *PVT1* could be a candidate for targeting therapy or not, further in vivo investigations with a larger sample size and determining the C-MYC protein level is suggested.

Conclusion

The result of this study demonstrated that *PVT1* blockage by antisense LNA GapmeRs reduces cell viability and induces apoptosis and necrosis in bone marrow cancerous blast cells. Also, *PVT1* expression is correlated with *c-Myc* expression and *PVT1* inhibition could reduce the expression of *c-Myc* as well. Furthermore, *PVT1* blockage could be a potential strategy in AML-M0 treatments. However, more studies with larger sample sizes in different populations are required.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This study was approved by the local ethics committee of Isfahan University of Medical Sciences (IRAN) and the study has 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.

Informed consent Informed consent was obtained from all individual participants included in the study.

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