Inducing Apoptosis and Decreasing Cell Proliferation in Human Acute Promyelocytic Leukemia Through Regulation Expression of *CASP3* by Let-7a-5p Blockage

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Abstract MicroRNAs (miRNAs) are short and single strand non-coding RNAs that involved in post-transcriptional regulation of gene expression. Dysregulation of miRNA expression is important event in the many of malignant diseases. Up-regulation of Let-7a-5p expression in acute myeloid leukemia in human in previous studies was reported. In this study blockage of Let-7a-5p in human acute promyelocytic leukemia cell line (HL60) was done by using locked nucleic acid (LNA) method and subsequently expression of Let-7a-5p, cell proliferation, apoptosis, necrosis, and *CASP3* expression was measured. At three time points 24, 48 and 72 h after LNA anti-Let-7a-5p transfection, assessment of Let-7a-5p expression by qRT real-time PCR was completed. The MTT assay and annexin/PI staining have been performed. Also, *CASP3* expression at different time points after LNA anti-Let-7a-5p transfection in HL60 cell line was measured. The results at three-time points after LNA transfection were represented that Let-7a-5p expression was lower in the LNA-anti-Let-7a group compared to the control groups. The cell

viability significantly was different between LNA-anti-Let-7a group and control groups. Increasing apoptotic ratio was associated with Let-7a-5p blockage in the LNA-anti-Let-7a group compared with control groups. Also, the necrotic ratio was higher in the LNA-anti-Let-7a group rather than the other groups. Western blotting revealed that *CASP3* expression associated with Let-7a-5p inhibition. Our results displayed that blockage of Let-7a-5p can reduced cell viability mainly due to the induction of apoptosis and *CASP3* up-regulation in HL60 cells. These results can be useful in translational medicine for research of antisense therapy in leukemia.

Keywords Let-7a-5p · Locked nucleic acid · Acute promyelocytic leukemia · *CASP3* · miRNA

Introduction

Acute promyelocytic leukemia (APL) is the one of a severe type of acute leukemia with fast onset symptoms, disseminated intravascular coagulation (DIC), poor response to chemotherapy and high mortality rate [1]. The most common cause of APL is a chromosomal translocation on chromosome 17 in retinoic acid receptor alpha (*RARα*) gene and the acid promyelocytic leukemia gene on human chromosome 15 [2]. Despite of many studies on APL, some aspect of biological and pathogenesis features of this leukemia have not been completely clear. Recent advances in the molecular biology and molecular medicine identified factors that involving in the regulating of gene expression predominantly the role of miRNAs. miRNAs are non-coding and small single strand RNA about the 19–25 nucleotides and plays an important role in different biological processes such as growth, differentiation, cell death

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as well as inflammation [3]. miRNAs genes transcript by RNA polymerase II and produce a primary miRNA as pri-miRNA. pri-miRNA has been hundred to several thousand nucleotides in which the stem-loop structures can be observed and contains a Cap at the end of the 5' and 3' poly-adenylate structure. The pri-miRNA molecule cut by Drosha enzyme into the nucleus and produce a pre-miRNA. Pre-miRNA by Exportin 5 Ran-GTP-dependent is transported to the cytoplasm and cutting by an RNase III enzyme called Dicer and produces a double-stranded miRNA. MiRNA strand of the duplex miRNA/miRNA*, transmitted into the RNA-induced silencing complex (RISC) and miRNA* is lost. The main activity of RISC complex is directed miRNA to the mRNA target. miRNA binds to the 3' UTR of mRNA and block the production of proteins [3–5].

According to the advances in gene therapy and non-coding RNA blockage, perhaps found the solution to the treatment of acute myeloid leukemia and the results are also used to treat other malignancies. The previous studies were reported that miRNAs plays a main role in the normal blood-forming hematopoietic through control expression of key importance such as transcription factors, growth factor receptors and chemokine receptors [6, 7]. Association between abnormal expression of miR-15 and miR-16 in chronic lymphocytic leukemia (CLL) has been reported [8]. Schotte et al. showed in ALL fourteen miRNAs were overexpressed include miR-128a, miR-142-3p, miR-142-5p, miR-150, miR-181a, miR-181b, miR-181c, miR-193a, miR-196b, miR-30e-5p, miR-34b, miR-365, miR-582, miR-708 and five miRNAs include miR-100, miR-125b, miR-151-5p, miR-99a, let-7e were downregulated compared with normal CD34⁺ cells [9]. MiR-17-92 cluster in chronic myelocytic leukemia (CML) CD34⁺ cells in chronic phase has been overexpressed [3].

Overexpression of let-7a miRNA is a common occurrence in AML and is associated with poor clinical outcome [10]. The Let-7a miRNA suppresses drug-induced apoptosis by targeting of CASP3 [11]. The family of let-7a has been appeared as a tumor-suppressor miRNAs in several studies due to their down-regulation with the up regulation of oncogenes genes such as RAS and HMGA2 [12, 13]. Additionally, the let-7 modulates cell proliferation pathways and this dysregulation is common event in cancer cells [14]. Bioinformatic analysis revealed the potential recognition sequences of let-7a on the 3' untranslated region (3'UTR) of caspase-3 [11].

Caspase-3 is a protein caspase that encodes by the gene *CASP3* which is located at 4q34. *CASP3* is a member of the family proteins known as cysteine proteases that play an important role in the executive phase. Caspase-3 is active in both extrinsic (ligand-dependent death) and intrinsic (mitochondrial) cell death pathway [15].

The results of our study suggested that Let-7a-5p blockage has an anti-proliferation outcome in the HL60 cell line. Blockage of Let-7a-5p can be used by locked nucleic acid (LNA) that is a modified oligonucleotide with enzymatic resistance and high stability [16, 17]. The LNA anti- Let-7a suggested as a suitable method for therapeutic purpose due to their efficacy and safety [17, 18]. In the present study, LNA was used for Let-7a-5p blockage and their effect on cell proliferation, apoptosis, necrosis and *CASP3* expression in the HL-60 cell line was assessed.

Materials and Methods

Bioinformatics Analysis for Let-7a Target Genes Prediction

Using bioinformatics software includes Targetcan, Pictar, miRDB and miRWalk predicted that *CASP3* is a target of Let-7a. On the other hand, using Mimirna software confirmed that Let-7a has high expression in HL60 cell line.

Cell Culture

Human APL cell line (HL60) was purchased from National Cell Bank of Iran (Pasteur Institute, Tehran, Iran). The cells were maintained in RPMI 1640 medium (Gibco, Paisley, UK). This media was complemented with fetal bovine serum (FBS; Gibco) 10% v/v, GM-CSF 10 ng/ml (R&D Systems, Minneapolis, MN, USA), 100 µg/ml of streptomycin (Sigma-Aldrich, Saint Louis, MO, USA) and 100 U/ml of penicillin in the 25-cm² culture flasks with 5% CO₂ (Nunc, Roskilde, Denmark) at 37 °C. For the maintenance of cells in the exponential phase, twice a week passaged.

Cell Transfection

The nucleotide sequence of the has-Let-7a-5p was obtained from www.mirbase.org site as 5'-UGAGGUA-GUAGGUUGUAUAGUU-3' (MIMAT0000062). The miRCURY LNA microRNA Inhibitor for hsa-Let-7a-5p silencing and microRNA inhibitor negative control (scrambled) was purchased from the Exiqon company (Copenhagen, Denmark). The oligonucleotides of LNA and scrambled was labeled at the 5' end with a fluorescent dye (6-FAM). The HL60 cell line transfection was performed by PolyFectTM transfection reagent kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. 5 × 10⁵ cells in the exponential phase were seeded in six-well culture plates with 1.8 ml RPMI 1640 per well without FBS and antibiotics. Forty pm miRCURY LNA microRNA Inhibitor along with 4 µl PolyFectTM was

mixed in 200 µl Opti-MEMI Medium (Gibco, Paisley, UK) and incubated for 15 min at room temperature. The complex as rotational was added to the cells. After 6 h incubation at 37 °C, FBS and antibiotics were added, then the HL60 cells have been incubated for 24, 48 and 72 h. The cell transfection was evaluated by fluorescence microscopy and flow cytometry because the LNA were labeled with fluorescence dye (6-Carboxyfluorescein).

Reverse Transcriptase microRNA Real-Time PCR

MicroRNA real-time PCR were performed to the determination of Let-7a-5p inhibition efficiency by LNA-anti-Let-7a. The total RNA extraction was completed with miRCURY RNA Isolation Kit (Exiqon, Copenhagen, Denmark) after 24, 48 and 72 h after transfection and subsequently cDNA synthesis was done by Universal cDNA Synthesis Kit (Exiqon, Copenhagen, Denmark). The Real-time PCR method was performed by using SYBR Green Master Mix Kit (Exiqon, Copenhagen, Denmark) and specific primers for Let-7a-5p obtained from Exiqon. The ABI Step One Plus (Applied Biosystems, Foster City, CA, USA) instrument and $\Delta\Delta C_t$ method were used for data analysis of qPCR experiments.

Cell Viability Assay

The MTT (3-[4, 5 dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) assay was used to determine cell viability. In the live cells, MTT is a reduction by mitochondrial dehydrogenase and produce purple formazan. This change is directly associated with the number of living cells. The MTT assay was performed at the 24, 48 and 72 h post transfection. 200 µL MTT (Sigma-Aldrich, Saint Louis, MO, USA) with 50 mg/ml concentration was added to the 5×10^5 HL60 cells suspension in 2 ml RPMI 1640 medium and then 4 h incubated at 37 °C in the dark. Subsequently, 200 µL dimethyl sulfoxide (Sigma-Aldrich, Saint Louis, MO, USA) was added to per well and shake for crystals dissolution. One well as a blank sample prepared using exactly the same procedure. Absorbance was calculated at 570 nm by spectrophotometer (PG Instrument T80, PG Instrument, Leicestershire, England). Reading was transformed to the percentage of controls.

Apoptosis and Necrosis Assay

Apoptosis and necrosis in HL60 cells were detected by FITC Annexin-V apoptosis detection Kit with propidium iodide (PI) (Bio legend, San Diego, USA). Annexin-V detected phosphatidylserine on apoptotic cells and PI

staining was used for identification of necrotic cells (untreated cells were used for control). The procedure was performed at the 24, 48 and 72 h after transfection according to the manufacturer's guidelines and the cells were assessed by FACS Calibur Flow Cytometer (BD, California, USA).

Reverse Transcriptase CASP3 Real-Time PCR

The expression level of *CASP3* was assessed by real-time qPCR. Total RNA extraction and cDNA synthesis were performed by miRCURY RNA Isolation Kit and Universal cDNA Synthesis Kit from Exiqon (Copenhagen, Denmark). The *CASP3* gene expression was done by SYBR Green Master Mix Kit (Exiqon, Copenhagen, Denmark) and *GAPDH* gene act as internal control and normalization and primers including: *CASP3* F: 5' TCCACAGCAC CTGGTTATTATTC 3', R: 5' ACTCAAATTCGTGTTGCC ACCTTTC 3' and *GAPDH* F: 5' GGTGTGAACCATGA GAAGTATGA 3' R: 5' GAGTCCTCCACGATACCAA AG 3'. The ABI Step One Plus (Applied Biosystems, Foster City, CA, USA) instrument and $\Delta\Delta C_t$ method were used for data analysis of qPCR experiment.

Protein Extraction and Western Blot Analysis

Western blot analysis was done for determination of CASP3 protein concentration in HL60 cells. Protein extraction was performed by Cell Lysis BufferTM (Cell Signaling TECHNOLOGY, Danvers, MA, USA) according to the manufacturer's instructions. Protein concentration was measured by Quick StartTM Bradford Protein Assay (BIO-RAD, Hercules, CA, USA). Detection of molecular weight for CASP3 was performed by solubilized proteins (100 µg) that resolved on 10% SDS-PAGE with SpectraTM Multicolor Broad Range (Thermo SCIENTIFIC, Waltham, MA, USA) as a molecular ladder. Western blotting was done by electro blotting (BIO-RAD, Hercules, CA, USA) on nitrocellulose membrane (Amersham, Piscataway, NJ, USA).

The nitrocellulose membranes were probed with primary antibodies: mouse monoclonal antibody-anti human CASP3 (Santa Cruz Biotechnology, USA) and mouse monoclonal antibody-anti human GAPDH (Santa Cruz Biotechnology, California, USA) at 1: 250 dilution. Secondary antibody was goat anti-mouse IgG-HRP (Santa Cruz Biotechnology, California, USA) at 1:2,500 dilution. Immunoreactivity was detected by activating of HRP enzyme by Opti-4CN Substrate Kit (BIORAD, Hercules, CA, USA). Concentration of CASP3 protein was measured by ImageJ (version 1.4o) software (National Institute of Health, Bethesda, MD, USA).

Fig. 1 HL60 cells were transfected with 6-FAM labeled LNA-anti-Let-7a-5p and then assessed the transfection efficacy. The cells were detected by fluorescent microscope Phase contrast (a) and fluorescent (b). Images of the same field of HL60 cells show that the majority of the cells are transfected

Statistical Analysis

All tests were done three times. The results analyzed by SPSS version 20 (IBM, New York, NY, USA) software. The two-way analysis of variance (two-way ANOVA test) and also post hoc test were used for data analysis. Data were presented as mean $\pm$ SD. Statistical significance was defined as $P < 0.05$.

Results

Cell Transfection

The transfection efficiency was determined by fluorescence microscopy because the all of the oligonucleotides has been labeled with fluorescence. Transfection efficiency was approximately 85% (Fig. 1).

Fig. 2 Let-7a expression level by qRT PCR at 24, 48 and 72 h after LNA-Let-7a transfection. Data analysis was performed by $\Delta\Delta C_t$ method and untreated group (control) was considered as reference for all three-time points. Data are mean $\pm$ S.D of triple independent tests ($P < 0.031$)

Assessment Expression of Let-7a-5p in HL60 Cell Line

The expression of Let-7a-5p was evaluated by RT micro-RNA real-time PCR in HL60 cells transfected with the LNA-anti-Let-7a group, scrambled group and the untreated group at 24, 48 and 72 h after transfection. The expression level of Let-7a-5p was prominently lower in the LNA-anti-Let-7a group compared to the control groups at all three-time points ($P < 0.031$). The expression of Let-7a-5p at 24 h post transfection was the lowest level and gradually upper at the next two time points (Fig. 2).

Cell Viability Evaluation of HL60 Cell Line

The MTT assay was performed at 24, 48 and 72 h after transfection for assessment effects of Let-7a-5p blockage

Fig. 3 The cell viability was assessed by MTT assay at 24, 48 and 72 h after LNA-Let-7a transfection. The cell viability in control cells at all three-time points has been considered as 100% and the cell viability in other groups is represented as a percentage of control cells at the same time points. Data are mean $\pm$ S.D of triple independent tests ($P > 0.011$)

Fig. 4 Apoptosis assay was performed by annexin-V-PI staining at 24, 48 and 72 h after LNA-Let-7a transfection. Data shown in the graph are mean $\pm$ S.D of triple independent tests ($P < 0.015$)

on cell viability in HL60 cell line. The cell viability was revealed considerably decreased after LNA-anti-Let-7a transfection. The cell viability in HL60 cells transfected with LNA-anti-Let-7a gradually upper over time and were less than 46% at 72 h after transfection. The cell viability was lower in the scrambled group compared with the untreated group at all three-time points but was not significant and probably due to toxicity of transfection reagent. Different in cell viability was statistically significant between LNA-anti-Let-7a compared to scrambled and untreated group ($P > 0.011$) in all three-time points after transfection (Fig. 3).

Apoptosis and Necrosis Assessment in HL60 Cell Line

Using the Annexin-V and PI staining, the effect of Let-7a-5p blockage on apoptosis and necrosis in HL60 cells was assessed at 24, 48 and 72 h after transfection. The apoptotic ratio in the HL60 cells lower increased at 24, 48 and 72 h post transfection by scrambled compared with untreated cells. This increasing was not significant and probably due to toxicity of transfection reagent. The results revealed apoptosis dramatically increased in the LNA-anti-Let-7a group compared with another group at all three-time points ($P < 0.015$; Fig. 4). Also, the necrosis assay of HL60 cells displayed that necrosis ratio lower increasingly at 48 and 72 h after transfection by scrambled compared to the untreated cells. This increasing was not significant and probably due to toxicity of transfection reagent. The results showed that the necrosis considerably increased in the

Fig. 5 Assessment of late apoptosis/necrosis assay was done by annexin-V-PI staining at 24, 48 and 72 h after LNA-Let-7a transfection. Data shown in the graph are mean $\pm$ S.D of triple independent tests ($P < 0.02$)

Fig. 6 CASP3 expression level by qRT PCR at 24, 48 and 72 h after LNA-Let-7a transfection. Data analysis was performed by $\Delta\Delta Ct$ method and untreated group (control) was considered as reference for all three-time points. Data are mean $\pm$ S.D of triple independent tests ($P < 0.015$)

LNA-anti-Let-7a group compared to other groups at all three-time points ($P < 0.02$; Fig. 5).

Expression Level of CASP3 Increased in HL60 Cells After Blockage of Let-7a-5p

The expression level of CASP3 was assessed by RT-qPCR in the HL60 cells transfected with the LNA-anti-Let-7a

group, scrambled and the untreated group at three-time points 24, 48 and 72 h after transfection. The *CASP3* expression was upper in the LNA-anti-Let-7a group compared with control groups at all three-time points ($P < 0.015$). The highest level of *CASP3* was 72 h after transfection (Fig. 6).

Increase of CASP3 Protein Expression in HL60 Cells After Inhibition of Let-7a-5p

The expression level of CASP3 protein was measured by western blotting assay in transfected cells by LNA-anti-Let-7a-5p, scrambled and untreated cells at 24, 48 and 72 h after blockage of Let-7a-5p in HL60 cells ($P < 0.027$). The GAPDH protein was used as an internal control and for normalization. Expression level of CASP3 protein in 48 h after LNA-anti-Let-7a-5p transfection is comparable with the GAPDH. Increasing level of CASP3 protein is continuing at 72 h after LNA-anti-Let-7a-5p transfection (Fig. 7).

Discussion

AML is a disorder of hematopoietic stem cells by blocking differentiation of hematopoietic cells, resulting in an increase of clonal neoplastic cells or cell blast. The malignant transformation of hematopoietic stem cells leads to loss of function of normal hematopoiesis and if untreated, leads to death [19]. Up or down expression of some miRNAs in AML disease in several studies previously were reported [20, 21]. While, the up-regulation of a small number of miRNAs for inducing leukemic transformation is necessary [22, 23], other miRNAs act as a tumor suppressor and down-regulated in hematopoietic malignancy [24–26]. On the other hand, the functional impact of some miRNAs and their targets on leukemic phenotypes still remain unclear [24].

Apoptosis is a most important mechanism for removing damaged cells that cannot be restored [27]. Therefore, the disorder in apoptosis can lead to impaired cell proliferation and apoptosis and lead to the development of cancer

Fig. 7 Evaluation expression of the CASP3 protein was performed by western blotting at 24, 48, and 72 h after transfection (a). Data shown in the graph (b) are mean $\pm$ SD of three independent tests ($P < 0.027$)

[27, 28]. The cells have been two main pathways leading to apoptosis that includes extrinsic (death ligand) and the intrinsic pathway (mitochondrial) [29, 30]. However, both pathways lead to the activation of the executive caspase 3, 6 and 7, that these are important mediators of apoptosis [15]. So, caspases play an important role in the executive phase and their association in a number of cancer cell and cancer cell lines has been displayed [28, 31–33].

The Let-7a-5p was extremely downregulated after LNA-anti-Let-7a transfection and confirmed by qRT-PCR. The MTT assay demonstrated that decrease in the cell viability associated with blockage of Let-7a-5p. The results confirmed using apoptosis and necrosis assay and shown apoptosis and necrosis were increased in the HL60 cells after LNA-anti-Let-7a transfection. Also, western blotting assay clearly displayed that Let-7a-5p targeted CASP3 in HL60 cells.

As a result, dysregulation of Let-7a-5p confirmed in leukemia and may be apoptosis deficiency in APL associated with change in CASP3 expression. LNA-anti-Let-7a-5p used in present study for inhibition of Let-7a-5p in HL-60 cells. Results of qRT PCR displayed that Let-7a-5p was almost completely down regulated after LNA-anti-Let-7a-5p transfection. On the contrary, CASP3 expression level increased in LNA-anti-Let-7a-5p group. Decrease in cell viability shown is associated with Let-7a-5p inhibition by MTT assay. Our results validated by apoptosis/necrosis assay and exhibited that LNA-anti-Let-7a-5p significantly raising apoptosis/necrosis ratio in HL60 cells. Our results shown that Let-7a-5p blockage can reduce cell viability in HL60 cell line partially due to induction of apoptosis through CASP3.

Accordingly, the apoptosis regulation of Let-7a through negative regulation of CASP3 using Let-7a blockage in APL can be posing as a therapeutic strategy. The therapeutic strategies base on miRNA blocking as a way for cancer therapy previously described [34, 35]. The LNA is a modified oligonucleotide with high stability and non-toxic characteristics that act as anti-miRNAs that used for miRNAs blockage [17].

Our study was shown the role of Let-7a-5p blockage using LNA-anti-Let-7a-5p as anti-proliferative in APL cells. The common method for APL treatment is chemotherapy. In some conditions chemotherapy were have not any effect on APL and cannot reduce cell proliferation. In the therapeutic methods for cancer treatment, miRNAs therapy reached the clinical trial phase and it's anticipated as a cure method for cancer treatment. All-Trans Retinoic Acid (ATRA) is a routine method for treatment of APL, but a subgroup of patients is not treated with ATRA [36]. In these circumstances perhaps combination therapy by LNA-anti-Let-7a and ATRA can be

therapy effect on AML. Although in vivo studies in the future need to assess the practicality of this strategy.

Conclusion

The results exhibited that Let-7a-5p blockage with LNA-anti-Let-7a-5p induces apoptosis, necrosis and decreases cell proliferation as well as increased CASP3 expression in HL60 cells and recommend Let-7a-5p blockage by LNA can be possibly used only or combined with other therapies for AML treatment. In vivo studies in the future need to evaluate the possibility of this strategy and this can be an open attitude in translational medicine.

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Compliance with Ethical Standards

Ethical Approval This study was approved by the local ethics committee of Isfahan University of Medical Sciences (IRAN) and the studies have been approved by the appropriate institutional and/or a 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.

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

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