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ORIGINAL ARTICLE

Inhibition of MicroRNA miR-222 with LNA Inhibitor Can Reduce Cell Proliferation in B Chronic Lymphoblastic Leukemia

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Abstract MicroRNAs (miRNAs) are small regulatory molecules that negatively regulate gene expression by base-pairing with their target mRNAs. miRNAs have contribute significantly to cancer biology and recent studies have demonstrated the oncogenic or tumor-suppressing role in cancer cells. In many tumors up-regulation miRNAs has been reported especially miR-222 has been shown to be up-regulated in B chronic lymphocytic leukemia (B-CLL). In this study we assessed the effected inhibition of miR-222 in cell viability of B-CLL. We performed inhibition of mir-222 in B-CLL cell line (183-E95) using locked nucleic acid (LNA) antagomir. At different time points after LNA-anti-mir-222 transfection, miR-222 quantitation and cell viability were assessed by qRT-real time polymerase chain reaction and MTT assays. The data were analyzed by independent *t* test and one way ANOVA. Down-regulation of miR-222 in B-CLL cell line (183-E95) with LNA antagomir decreased cell viability in B-CLL. Cell viability gradually decreased over time as the viability of LNA-anti-mir transfected cells was <47 % of untreated cells at 72 h post-transfection. The difference in cell viability between LNA-anti-miR and control groups was statistically significant ($p < 0.042$). Based on our findings, the inhibition of miR-222 speculate represent a potential novel therapeutic approach for treatment of B-CLL.

Keywords MicroRNA · miR-222 · Chronic lymphocytic leukemia · Locked nucleic acid

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Introduction

MiRNAs are a family of 19-25-nucleotide small RNAs that negatively regulate gene expression at the post-transcriptional level by binding to the 3′-untranslated region of target mRNA [1, 2]. miRNAs play essential roles in many normal biological processes involving cell proliferation, differentiation, growth and development, metabolism, aging and apoptosis [3–7]. Recently, the role of microRNAs (miRNAs) in cancer has received much attention, much evidences points to miRNAs functioning as tumor promoters (oncomirs) such as: miR-155 and miR-17–92 [8], or tumor suppressors (anti-oncomirs) such as: miR-15a, miR-16, and miRNAs of the miR-34 and let-7 families [8, 9]. miRNA alterations are effective on all six hallmarks of malignant cells; in CLL, the main effects

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are apoptosis, evasion, self-sufficiency in growth, and as recently found, stimulation of dissemination and angiogenesis [10].

Chronic lymphocytic leukemia (CLL) is a disease of older patients, and with most patients ≥65 years of age and having at least one major property [11, 12], CLL is of B cell origin, it occurs in two forms, indolent and aggressive [11]. The most frequency chromosomal abnormalities detected in CLL include deletion at 13q (55 %), 11q (18 %), trisomy 12 (12–16 %) and 17p (8 %) but molecular details still under investigation [13, 14]. MicroRNAs in CLL can function as oncogenes, tumor suppressors, or be used as markers for disease onset/progression for example, in indolent CLL, 13q14 deletions targeting miR-15/16 initiate the disease, while in aggressive CLL miR-181 targets the critical TCL1 oncogene [11, 15]. The relative expression levels of certain microRNAs correlate with prognostic indicators in CLL for example, leukemia-cell expression of zeta-associated protein of 70 kDa (ZAP-70), use of unmutated immunoglobulin heavy-chain variable region genes (IGHV) [16–19]. miR-221/222 are highly homologous miRNAs encoded in tandem on the X chromosome, Amplification or overexpression of miR-221/222 is reported in hematopoietic malignancies indicating that miR-221/222 could be potential therapeutic targets [20]. The direct association between miR-222 and B-cell malignancies is indicated, upregulation of human miR-222 was reported in aggressive forms of CLL, glioblastoma, and thyroid papillary carcinoma [20–22]. The miR-221/222 cluster is capable of modulating the expression of p27 gene, it is a regulatory loop that contributes to keeping CLL cells in resting condition [20].

In this study, we knockdown mir-222 by locked nucleic acid (LNA), then evaluation expression of mir-222 and cell viability in 183-E95 cell line at 24,48,72 h after inhibtion.

Materials and Methods

Cell Culture

The 183-E95 cell line was purchased from the National Cell Bank of Iran (NCBI; Pasteur Institute, Iran).The cell culture was maintained in Roswell Park Memorial Institute (RPMI) 1640 (Gibco, Paisley, UK) supplemented with fetal calf serum (FCS; Gibco, Paisley, UK) 15 % v/v, 100 U/mL of penicillin and 100 µg/mL of streptomycin (Gibco, Paisley, UK) in a humid and air-saturated atmosphere consisting of 5 % CO_2 inside 25-cm^2 culture flasks (Nunc, Roskilde, Denmark) at 37 °C. The cells were passaged twice a week to keep an exponential growth phase.

Cell Transfection

The nucleotide sequences of miR-222 were obtained from www.mirbase.org as GAG ACC CAG TAG CCA GAT GTA GCT. The miRCURY LNA microRNA Inhibitor™ for hsa-miR-222 and miRNA inhibitor negative control (scrambled) oligonucleotides were purchased from Exiqon, Denmark. Both oligonucleotides were labeled at the 5′ end with fluorescent dyes, 6-FAM, for subsequent detection of transfected cells.

183-E95 cell transfection was performed by the Lipofectamin RNAiMax transfection Reagent (Invitrogen Germany) according to the manufacturer instructions. Briefly, 5 × 105 cells in the exponential growth phase were cultured in six-well culture plates (Nunc, Roskilde, Denmark) containing 1.8 mL RPMI 1640 per well without antibiotics and FCS. The 30 pmol mercury locked nucleic acids (LNA) microRNA inhibitor™ was mixed with 5 µL Lipofectamin RNAiMax Transfection Reagent™ in 200 µl Opti-MEMI Medium™ (Gibco, Paisley, UK) and incubated for 15 min at room temperature. Then, the complex was added to the cells and swirled carefully for even distribution over the entire plate surface. After 8-h incubation, the FCS and antibiotics were added, and the cells were incubated for 24, 48, and 72 h. Untreated and scrambled-LNA transfected cells were cultured parallel to the LNAanti-miR transfected cells. Efficiency of the transfection was investigated by flow cytometry and fluorescent microscopy. LNA was conjugated with 6 FAM™ Fluorescein (6-carboxyfluorescein) to identify and quantify LNA transfected cells by fluorescent microscopy and FACSCalibur flow cytometer (Partec, Germany).

Reverse Transcriptase micRNA Real Time Polymerase Chain Reaction (PCR)

First the expression level of mir-222 in B-CLL cell line (183-E95) was determined by qRT-real time PCR. Reverse transcriptase (RT) miRNA real time PCR was done to determine the efficiency of miR-222 inhibition using LNA anti-miR. Briefly, the total cellular RNA was extracted 24, 48, and 72 h post-transfection with the miRCURY RNA Isolation Kit™ (Genall) and cDNA was synthesized with the Universal cDNA Synthesis Kit™ (Parsgengan). Real time PCR was performed by SYBR Green Master Mix Kit™ (Takara) and specific miR-222 primers (all consumables in this section were from Metabion, Germany). Synthetic RNA Snord 47 templates and their primers (Metabion, Germany) were used for real time PCR internal control. The ABI Step One Plus (ABI, USA) instrument was used for real time PCR experiments and the $2^{-\Delta\Delta Ct}$ method for data calculation.

Cells Viability Assay

The viability of cells was determined by the MTT (3-[4, 5dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) assay, based on reduction of MTT by the mitochondrial dehydrogenase of intact cells to purple formazan products. The conversion is directly related to the number of living cells. The MTT assay was performed at the intervals of 24, 48, and 72 h post-transfection. Two hundred µl of MTT (Sigma-Aldrich, USA) at the concentration of 50 mg/mL was added to 5 × 105 183-E95 cells suspended in 2 mL of RPMI 1640 medium and incubated for 4 h at 37 °C in the darkness. Two hundred µl of dimethyl sulfoxide (Sigma-Aldrich, USA) was added to each well and was shaken until dissolution of crystals. Blank samples were prepared per exactly the same procedure except for cell incorporation. Absorbance was measured by a spectrophotometer at 570 nm. Reading was converted to the percentage of the controls.

Statistical Analysis

Statistical analyses were performed using SPSS version 16.0. independent t test and one-way ANOVA was used for the analysis of the differences between groups. A p value of <0.05 was considered statistically significant. All experiments were replicated three times.

Results

miRCURY LNA microRNA Inhibitor™ powerfully Inhibits expreation of miR-222

The up-regulation of miR-222 was showed in B-CLL cell line (183-E95) by qRT-real time PCR. For inhibition of miR-222, the miRCURY LNA microRNA Inhibitor™ was transfected to 183-E95 cells with the Lipofectamin RNAiMax Transfection Reagent. On the basis of the initial optimization experiments, transfection was performed with 30 pM of LNA-anti-miR and 5 µl of the transfection reagent. As the transfected oligonucleotides were fluorochrome-conjugated, transfection efficiencies were assessed by fluorescence microscopy and flow cytometry. Carefully optimized protocol produced transfection efficiency of 80 % (Fig. 1).

Expression of miR-222 was assessed by reverse transcriptase microRNA real time PCR in 183-E95 cells transfected with the miRCURY LNA microRNA Inhibitor™ (LNA-anti-miR group); the microRNA inhibitor scrambled oligonucleotides (scrambled LNA group) and untreated 183-E95 cells (untreated groups) at 24, 48, and 72 h post-transfection. Although miR-222 expression was a little lower in the scrambled LNA-transfected cells compared to the untreated cells, the differences were not statistically significant. However, in all three intervals, the expression of miR-222 was considerably lower in the LNA-anti-miR group compared to the control groups ($p < 0.0037$). The expression of miR-222 was at the lowest level at 48 h after transfection (Fig. 2).

Expression of miR-222 in B-CLL cell line (183-E95) in three groups:, scrambled, LNA- anti-mir222 and untreated determined by quantitative RT-PCR. Numeral upper columns is p value result of compared between groups. Values shown represent the mean ± SD.

Inhibition of miR-222 Decreased Viabiliy 183-E95 Cells

To assess the effect of miR-222 inhibition on cell viability, the MTT assay was performed at 24, 48, and 72 h post-transfection. Cell viability was lower in LNA-anti-miR group compared to the untreated cells at 48 and 72 h post-transfection ($p < 0.05$). The effect of LNA-anti-miR transfection on the cell viability increased over time as the viability of the LNA-anti-miR transfected cell was <47 % of the untreated cells at 72 h post-transfection. The difference in the cell viability between the LNA-anti-miR and both control groups (untreated and scrambled treated) was statistically significant ($p < 0.042$) at all three intervals (Fig. 3).

The viability of the untreated cells in each time point was considered as 100 % and the viability of other groups is presented as the percentage of the untreated cells in the same time point. Data were mean ± SD of three independent experiments.

Discussion

B-cell chronic lymphocytic leukemia (B-CLL), also known as chronic lymphoid leukemia (CLL) is a type of cancer of the white blood cells in adult [5, 23]. CLL has a variable course, Some patients require little or no treatment, but in some other patients, the disease is progressive and results in the death despite various treatments, Effective choices of treatment are available for CLL patients, including chemotherapy and monoclonal antibodies [24, 25].These types of treatments can induce remissions in up to 90 % of patients. Some patients, however, do not respond to up-front chemoimmunotherapy combinations, or they develop resistance to the treatment quickly. Despite recent progresses in treatment of this disorder, because of the effects of chemotherapy and radiotherapy, including adverse effects on normal cells, seeking for new therapies seems necessary. In the recent years, it has been demonstrated that

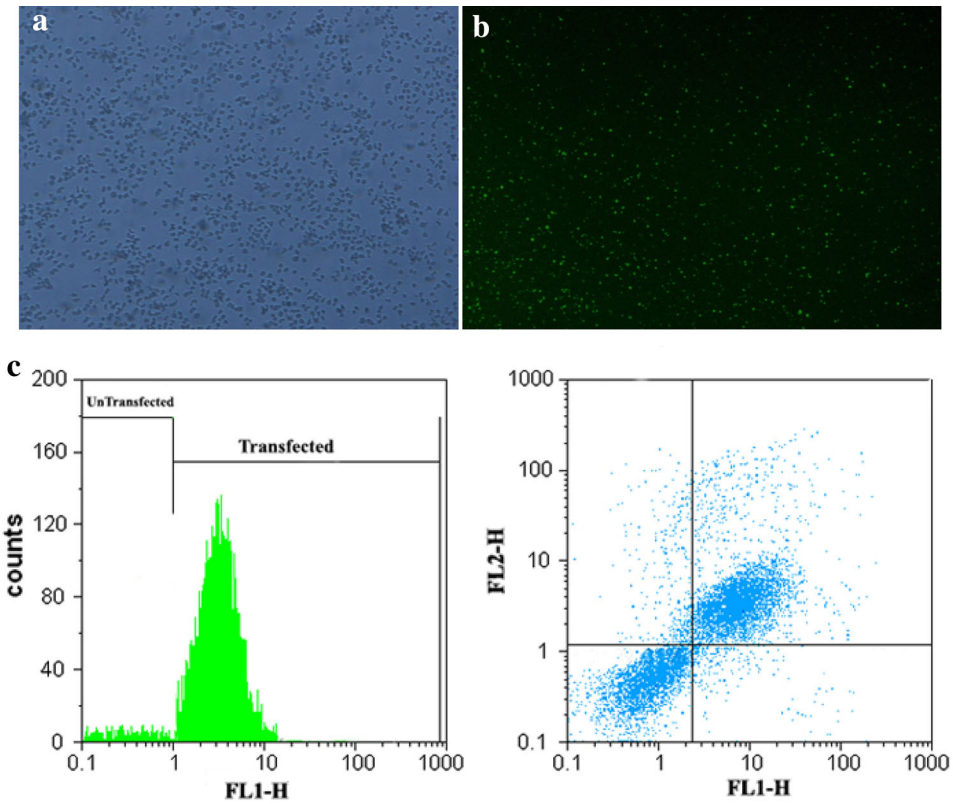

Fig. 1 Transfection miR-222 show with fluorescent microscope and flow cytometry 183-E95 cells have been transfected with 6-FAM™ fluorescein-conjugated LNA oligonucleotides and then to assess transfection efficiency, they analyzed by flow cytometry and have been observed with a fluorescent microscope. Phase contrast (**a**) and fluorescent (**b**) images of the same field of 183-E95 cells show that a majority of the cells were transfected. Representative FSC-SCS and FL1-Count flow cytometry graphs are shown in **c**. *Scale bars* 50 μm

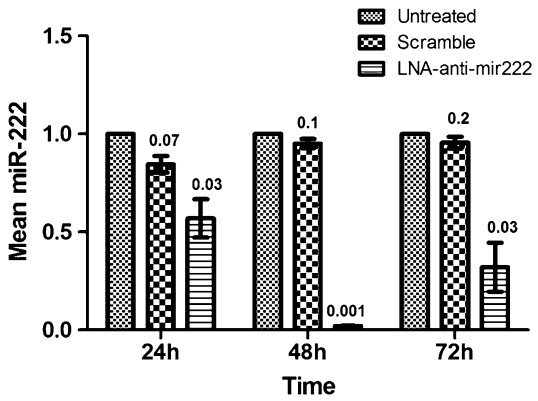

Fig. 2 Evaluation of miR-222 by qRT-PCR performed 24, 48, and 72 h after trasfection

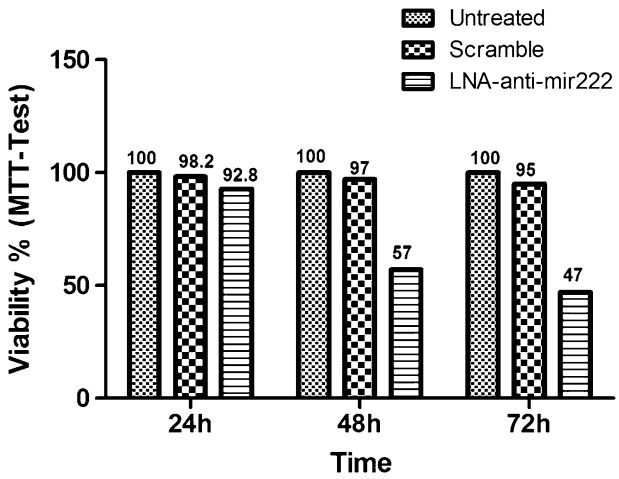

Fig. 3 Evaluation of cell viability by the MTT assay performed 24, 48, and 72 h after transfection

small regulatory non-coding RNAs (ncRNAs) named microRNAs (miRNAs) are involved in human tumorigenesis, Since miRNAs can function as either oncogenes or tumor suppressor genes in tumorigenesis, the potential of using these small RNAs as therapeutic targets opens up new opportunities for cancer treatments by either inhibiting or augmenting their activity [26].

Among miRNAs significantly deregulated in human cancer, miR-221/222 are of major interest potential targets for therapeutic application. miR-221/222 act as oncogenic miRNAs that as facilitate cell proliferation via down-regulation of $p27^{KIP1}$ and/or $p57^{KIP2}$ [27], as potential targets for therapeutic applications, which negatively regulate cell cycle progression from G1- to S-phase [28]. p27 binds to cyclin-dependent kinase 2 and cyclin E complexes to prevent cell-cycle progression from G1 to S phase. Several reports suggested a key role of miR-221/222 in tumorigenesis. In addition, other authors recently showed that miR-

221/222 antisense oligonucleotides reduce tumor growth by increasing intra-tumor $p27^{KIP1}$ protein expression [29].

A evidence suggests that silencing microRNAs with oncogenic potential may represent a successful therapeutic strategy for human cancer. A new therapeutic strategy to inhibit miRNAs that are involved in the pathogenesis of the disease, which is the aim of the present study, we can use engineered nucleic acid (LNA) that create a stable connection with the target miRNA and are resistant to nucleases. They particularly disrupt the performance of specific molecule (miR) which plays a role in prevalence of cancer [30, 31]. In this study we used LAN-anti-mir to inhibit mir-222 in B-CLL in cell line 183-E95, Prevention of cellular proliferation subsequent to the LNA transfection is indicative of successful inhibition of miR-222 in the cell line under study. Real time PCR confirmed our data and miRNAs reduced expression almost by transfection of LNA. MTT test showed that inhibition of mir-222 caused reduction of viability cell after 24, 48 and 72 h. Cell viability had the minimal reduction in LNA-scrambled and in comparison to untreated cells with no significant difference.

There are many articles related to oncomir that show treatment with this method is used for many types of cancers. Inhibition effect of miR-221 by anti-miRNA oligonucleotide in CLL p53-mutant MEG-01 cells, significant increase in caspase activity in fludarabine-treated in cell line [32]. The up expression of miR-221/222 in the CLL cell line MEC1 promote down-regulation of p27 protein therefore miR-221/222 and p27 may exhibit a regulatory loop that helps maintaining CLL cells in a resting condition [20]. The up expression of miR-92a in APL cell line (HL-60) by LNA antagomir extensively decreased cell viability [33]. The difference of cell viability between LNA-anti-miR and control groups was statistically significant ($p < 0.024$). In a study by Di Martino et al. [34] on the in vitro effect of inhibiting miR-221/222 on cancer cell multiple myeloma, LNA technology was used to inhibit miR-221/222. Their studies showed that by inhibiting miR-221/222 in cell multiple myeloma, $p27^{KIP1}$, PUMA and PTEN genes that all have the role of tumor suppressor in cell cycle are increased but decreased in reverse cell proliferation. More recently, it has been reported that the treatment with LNA-modified miR-221 inhibitors reduces the growth of liver cancer cells over-expressing miR-221/222 in vitro by targeting a DNA damage-inducible transcript 4 (DDIT4), a modulator of the mTOR pathway [35].

Chemotherapy is a routine treatment in CLL. But, different findings have been obtained in cases resistant to treatment. On the other hand, there are some instances that oncomiR inhibition acts to sensitize the cells to chemotherapy agents, making this combination therapy influenced more than any one of the strategies used alone. Our data suggest that inhibition of miR-222 with LNA-anti-miR may provide an alternative approach for the treatment of B-CLL. It can be used alone or in combination with current therapies to reduce the existing limitations in the treatment of this malignancy.

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Compliance with Ethical Standards

Conflict of interest The authors have no conflicts of interest, including financial interests, relationships, and/or affiliations relevant to the subject matter or materials included.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

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