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Decreased cell proliferation and induced apoptosis in human B-chronic lymphocytic leukemia following miR-221 inhibition through modulation of p27 expression

Korosh Ashrafi Dehkordi[1], Majid Asadi-Samani[1,4], Ali Shojaeian[2] and Mohammad-Reza Mahmoudian-Sani[3*]

Abstract

Background: This study aimed to investigate the effects of the miR-221 inhibition on the human B-chronic lymphocytic leukemia (B-CLL) cell viability and the p27 gene expression, to introduce a new treatment approach for this type of cancer. In this context, the cyclin-dependent kinase (Cdk) inhibitor 1B ($p27^{Kip1}$) is considered as an enzyme inhibitor that encodes a protein belonging to the Cip/Kip family of the Cdk inhibitor proteins.

Methods: The affected miR-221 inhibition in the B-CLL cell viability was initially assessed. The inhibition of miR-221 in the B-CLL cell line (183-E95) was thus performed using locked nucleic acid (LNA) as an antagomir. After the LNA-anti-miR-221 transfection, the miR-221 quantification, cell viability, and apoptosis assays were evaluated at different intervals by the reverse transcription-quantitative polymerase chain reaction (RT-qPCR), the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay, and flow cytometry (FC), respectively. The qRT-PCR was also completed for the p27 gene. The data were subsequently analyzed by independent-samples *t*-test and one-way analysis of variance (ANOVA).

Results: A gradual reduction was observed in the B-CLL cell viability, and consequently the transfected LNA-anti-miR cell viability reached below 55% of the untreated cells after 72 h of transfection. A statistically significant difference was found in the cell viability between the LNA-anti-miR-treated and control groups (p-value ≤ 0.043). The downregulation of miR-221 in the B-CLL (183-E95) cells was further conducted by LNA-anti-miR-221.

Conclusion: The miR-221 inhibition significantly decreases cell viability through augmenting the p27 gene expression and inducing apoptosis. Moreover, the findings demonstrated that the inhibition of miR-221 might be a new treatment approach for B-CLL, although more confirmation is needed by investigating appropriate animal models.

Keywords: Chronic lymphocytic leukemia, MicroRNA, MiR-221, Locked nucleic acid

Background

MicroRNAs (miRNAs) are a group of small 19–25 RNA nucleotides that help downregulate the post-transcriptional expression of genes by linking to the 3'-untranslated regions (3'-UTRs) of the target mRNA. They further contribute to different biological and natural processes, like cell growth and development, proliferation, differentiation, aging, metabolism, and death [1]. Currently, much attention has been attracted to miRNAs in cancer. In this context, evidence shows that miRNA serves as a tumor promoter, and above all plays the role of an oncomiR, including miR-17-92 and miR-155 [2], or an anti-oncomiR (as a tumor suppressor), such as lethal-7

*Correspondence: mohamadsani495@gmail.com

[3] Thalassemia and Hemoglobinopathy Research Center, Health Research Institute, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
Full list of author information is available at the end of the article

(let-7), miR-34, miR-16, and miR-15a families [2, 3]. The changes in the expression of miRNAs are thus of utmost importance in the development of malignant cells in CLL evasion, apoptosis, self-sufficiency in growth, and induced angiogenesis and dissemination as the main effects [4]. CLL can be accordingly deemed as a disease in adults, affecting the majority of the patients aged over 65. This condition develops from the type B cells, and is categorized into aggressive and indolent groups [5]. Although genetic investigations have partially reflected on the CLL etiology, molecular details are shrouded under mystery. For instance, the main chromosomal abnormalities have been observed in CLL, including deletion at 13q (55%), 11q (18%), trisomy 12 (12–16%), and 17p (8%) [6, 7]. The fact is that miRNAs in CLL can serve as oncogenes, i.e., tumor suppressors, which can be implied as the biomarkers for the onset and progression of the disease. Reportedly, the deletion of the 13q14-targeting miR-15,16 in the indolent CLL causes the onset of disease, while miR-181 targets the susceptible cancer gene TCL1 in the aggressive CLL [5, 6, 8]. The expression levels of some miRNAs are related to those of certain markers, such as zeta-associated protein of 70 (ZAP70) and the regions of unmutated immunoglobulin heavy-chain variable (IgHV) gene [6]. MiR-221/222 is also encoded on chromosome X, whose over-expression has been found in hematologic malignancies, indicating the miR-221/222 function in the CLL pathogenesis [9]. MiR-221 is further directly correlated with the B-cell-related malignancies, and its expression has been reported to increase in the aggressive CLL, glioblastoma (GBM), and thyroid papillary carcinoma (TPC) [9–11]. In this line, the p27 gene expression is controlled by the miR-221/222 cluster through the regulation of the CLL cells in a resting state [9].

One of main challenges in the pharmaceutical industry is introducing new treatments to reduce the risk of cancer. Nowadays, more understanding of the genetic and molecular roots of cancer can be helpful to develop new-targeted treatments, such as antisense therapy, which refers to the application of the single-stranded oligonucleotides with therapeutic purposes. Locked nucleic acid (LNA), as a new RNA analogue, also possesses many features essential for fabricating a strong, safe antisense drug [12]. Thanks to their unprecedented RNA binding characteristics, oligonucleotides, such as the LNA nucleotides are assumed good candidates to silence the genes, and many successful LNA antisense studies have already been the subject of detailed reviews. For instance, this approach has been successfully applied for the CLL patients [13]. In the present study, miR-221 in the 183-E95 cell line was thus inactivated by LNA, to subsequently evaluate the impacts observed on the cell proliferation, apoptosis, and p27 gene expression levels. Although further in vivo examinations are needed, the study findings can have implications as a new potential approach to treat CLL.

Methods

Cell culture

The cells (viz. 183-E95 cell line, NCBI-Iran, Catalog no. C492) were cultured onto the Roswell Park Memorial Institute (RPMI) 1640 medium, added by 15% v/v fetal calf serum (FCS). In addition, streptomycin (100 µg/ml) and penicillin (100 U/ml) (Gibco, Paisley, UK) in a wet medium with 5% carbon dioxide (CO_2) were maintained at the temperature of 37 °C in specific flasks with 25 cm^2 area. The cells were then kept at their exponential growth phase because of weekly twice passages.

Cell transfection

The miR-221 sequence (GAAACCCAGCAGACAAUG UAGCU) was extracted from http://www.mirbase.org. The miRNA inhibitor as a negative control (scrambled) and the miRCURY™ LNA miRNA inhibitor for hsa-miR-221 were further acquired from Exiqon (Denmark). In addition, the transfected cells were identified by the oligonucleotides labeled 5' with the 6-carboxyfluorescein (6-FAM) dyes. The Lipofectamine RNAiMAX™ transfection reagent (Invitrogen, Germany, Cat. no. 13778030) was further applied for the 183-E95 cell transfection based on the related protocol. Thus, a density of 5×10^5 cells was added to a 6-well petri dish (Nunc, Roskilde, Denmark) in the exponential growth phase in the presence of the RPMI 1640 (1.8 mL of per well) with no antibiotics and FCS. After adding the miRCURY™ LNA miRNA inhibitor (30 pmol) to the Lipofectamine RNAiMAX™ transfection reagent (5 µL) in the Opti-MEM Medium™ (200 µL) (Gibco, Paisley, UK), the incubation was performed at an ambient temperature for 15 min, which was then appended by the cells to cover the whole plate surface, and incubated for 8 h. Afterward, the antibiotics and FCS were added, and the incubation was completed at different intervals of 24, 48, and 72 h. Simultaneously with the LNA-anti-miR transfected cells, the culture continued for the untreated and scrambled-LNA transfected ones. Fluorescence microscopy (FM) and flow cytometry (FC) were consequently applied to evaluate the transfection efficiency. The LNA-conjugated 6-FAM was also employed to determine and count the LNA-transfected cells via a flow cytometer and a fluorescent microscope (Partec, Germany).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Firstly, the RT-qPCR technique was recruited to evaluate the miR-221 expression level in the B-CLL cell line

(183-E95) and analyze the miR-221 inhibition efficiency via the LNA anti-miR, and then the p27 gene expression at different intervals of 24, 48, and 72 h following inhibition. Thus, the extraction of the total cell RNA was performed after 24, 48, and 72 h of transfection using miRCURY RNA Isolation Kit™ (GeneAll, Seoul, Korea), followed by the complementary DNA (cDNA) construction via the Universal cDNA Synthesis Kit™ (Parsgengan, Tehran, Iran). Consequently, the PCR process was accomplished by exploiting the SYBR Green Master Mix Kit (Takara, Japan), the miR-221 primers, and the p27 gene, whose materials were from Metabion, Germany, and the Synthetic RNA Snord 47 templates and primers were considered as internal controls.

Cell viability assessment

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay was applied to measure cell viability, so that a reduction in MTT was the result of the intact cell conversion into the purple formazan products via mitochondrial dehydrogenase. This reaction was related to the count of the viable cells. After transfection, the MTT assay was carried out at different intervals of 24, 48, and 72 h. Thus, the MTT (Sigma-Aldrich, USA) at a concentration of 50 mg/mL (200 µl) was poured on 5×10^5 floating 183-E95 cells in the RPMI 1640 medium (2 ml), followed by incubation at 37 °C for 4 h in a dark condition. In addition, each well was appended by 200 µl of dimethyl sulfoxide (DMSO) (Sigma-Aldrich, USA) and stirred to dissolve the crystals. The blank samples were further prepared according to the same protocol; however, they had no cells. To determine the absorbance, a spectrophotometer at 570 nm wavelength was utilized.

Apoptosis assay

The apoptosis induction in the 183-E95 cells was evaluated using the Annexin V Staining Kit™ (Roche, Germany). Thus, the phosphatidylserine was detected by this kit in the apoptotic cells, and propidium iodide (PI) staining was performed for the necrotic cell discrimination. Based on the relevant protocol recommended for this purpose, staining was conducted after 24, 48, and 72 h of transfection. At this step, the control was considered as the untreated cells, and the analysis of the cells was performed via a flow cytometer with 500-nm excitation, 520-nm bandpass filter for the detection of fluorescein-conjugated Annexin V, and a 600-nm filter for the PI determination.

In silico *evaluation of miRNA binding sites*

The in silico analysis of the miRNA binding sites was compared in the genes via the online prediction tools of miRWalk, RegRNA 1.0, miRanda, and TargetScan4.2 (http://www.targetscan.org). The data showed the uniqueness of the miRNA binding sites and rolled out the off-targets.

Statistical analysis

The statistical analyses were conducted using the SPSS (Ver. 16) software package, exploiting independent-samples *t*-test and one-way analysis of variance (ANOVA) to investigate the inter-group differences. The significance level was further statistically estimated at p-value < 0.05. All the experiments were tested in triplicate.

Results

MiR-221 expression, potently inhibited by miRCURY LNA miRNA inhibitor™

The RT-qPCR findings indicated an increased level of the miR-221 gene expression in the B-CLL cells. Practically, the miRCURY LNA miRNA Inhibitor™ along with the Lipofectamine RNAi MAX Transfection Reagent™ was utilized to transfect the 183-E95 cells to inhibit miR-221. For the primary optimization tests, transfection was conducted by LNA-anti-miR (30 pM) and transfection reagent (5 µL). The fluorochrome-conjugated transfected oligonucleotide complexes were further assayed by FC to evaluate the transfection efficiency. The results of the transfection efficiency have been presented in our previous published paper [14]. According to our published paper, the transfection efficiency was determined around 80%. The miR-221 and the p27 expression levels were then detected in the 183-E95 cells with three treatment groups, including the untreated 183-E95 cells (untreated group), the miRNA inhibitor scrambled oligonucleotides (scrambled LNA group), and the miRCURY LNA miRNA Inhibitor™ (LNA-Anti-miR group) after 24, 48, and 72 h of transfection. There was a slight reduction in the miR-221 expression in the scrambled LNA-transfected cells as compared with the untreated ones, but their differences were not statistically significant. Nevertheless, the miR-221 gene expression was reduced in the LNA-anti-miR group at all three intervals than the controls (p-value ≤ 0.0027); in contrast, the p27 gene expression was elevated in the LNA-anti-miR group than the controls (Fig. 1A and B).

Decreased 183-E95 cell viability following miR-221 inhibition

The MTT assay was used to evaluate the impact of the miR-221 inhibition on cell viability after 24 and 48 h of transfection. The data indicated the loss of viability in the LNA-anti-miR group in comparison with the untreated groups after 24, 48, and 72 h of transfection (p-value < 0.05). The cell viability further grew after transfection with LNA-anti-miR over time, such that the

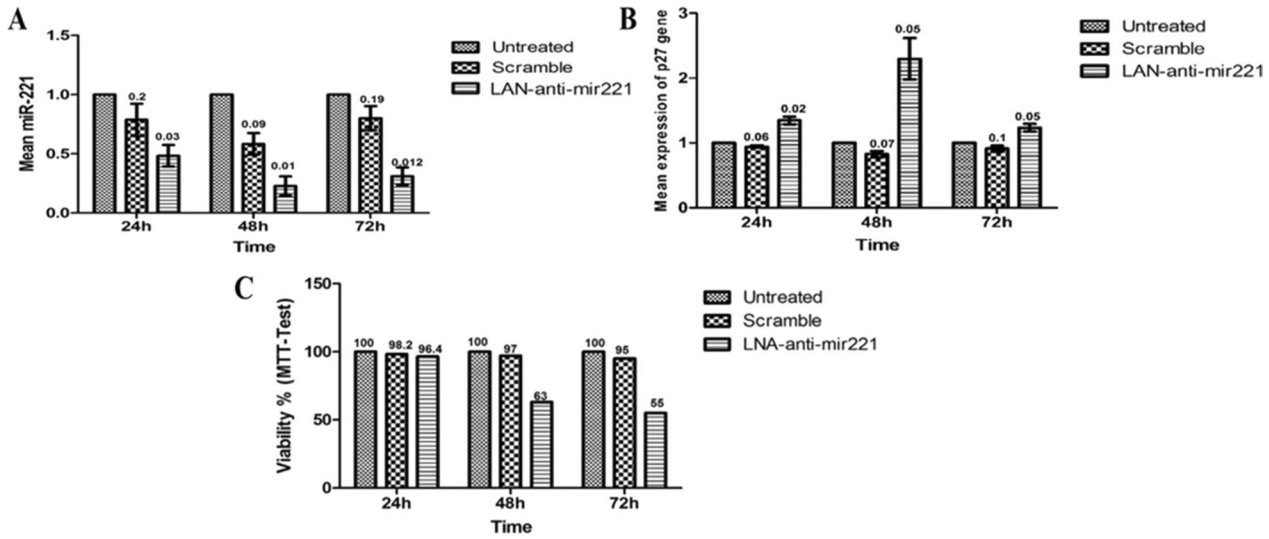

Fig. 1 **A** Evaluation of miR-221 by qRT-PCR performed at 24, 48, and 72 h after transfection. **B** Assessment of *p27 gene* expression by qRT-PCR performed at 24, 48, and 72 h after transfection. **C** Measuring cell viability by the MTT assay performed at 24, 48, and 72 h after transfection

LNA-anti-miR transfected cell viability reached < 55% of the untreated cells after 72 h of transfection. A dramatic difference was also observed in the cell viability percentage between LNA-anti-miR and both untreated and scrambled-treated groups (controls) at all three intervals (p-value ≤ 0.043) (Fig. 1C).

Increased apoptosis in 183-E95 cells following miR-221 inhibition

The possible effects of miR-221 were assessed on the Annexin V-PI-stained cell apoptosis after 24, 48, and 72 h of transfection. Of note, the untreated cells showed no apoptosis; however, the proportion of the cells moderately elevated once compared with the treated ones at 72 h following scrambled-LNA transfection. It seems that the miR-221 inhibition was associated with increased apoptosis because of the higher proportion of the apoptotic cells in the LNA-anti-miR group in comparison with other groups, at all three intervals (p-value ≤ 0.0071) (Fig. 2).

Discussion

B-CLL has been identified as a form of cancer affecting white blood cells, predominantly in adults [15]. Although various treatments, from chemotherapy to monoclonal antibodies, are being applied at the CLL stages, some patients are still at the risk of CLL progression [16]. These treatments result in improvements in around 90% of the cases; however, chemo-immunotherapy combinations fail to eradicate or arrest cancerous cells in some patients.

Despite the recent advances in the treatments for CLL, it seems indispensable to find novel approaches due to the complications from radiotherapy and chemotherapy on other normal cells. In recent years, miRNAs have been found to take a stage in tumorigenesis. Indeed, miRNAs play their oncogenic or tumor-suppressing roles here; thus, they can be utilized for therapeutic purposes, which provide new opportunities to treat cancer through the inhibition or augmentation of their activities [17]. Among the existing miRNAs, whose expression is significantly disrupted in different types of cancer in human, miR-221/222 has drawn the greatest attention to be examined for therapeutic potentials. Since the miR-221/222 targets are genes with a pivotal role in cancer, they cause a decline in apoptosis and an elevation in the p27 proliferation through the downregulation of $p27^{Kip1}$ and/or $p57^{Kip2}$ [18]. As well, $p57^{Kip2}$ can be considered as a regulatory factor for cell cycles from G1 to S phase [19]. Acting with therapeutic applications, p27 is accordingly linked with cyclin E and Cdk 2 complexes, to inhibit the cell cycle process. The miR-221/222 is more likely to regulate the tumorigenesis. Furthermore, the tumor growth has been demonstrated to decrease by the miR-221/222 antisense oligonucleotides through the rise in the expression of the intra-tumor $p27^{Kip1}$ protein [11]. The expression levels of $p27^{Kip1}$ and miR-221/222 are also inversely correlated in pancreatic cells. Furthermore, the miR221/222 over-expression has crucial consequences on cell proliferation and cell cycle distribution [20]. There are above all similar findings for GBM [21], TPC [22],

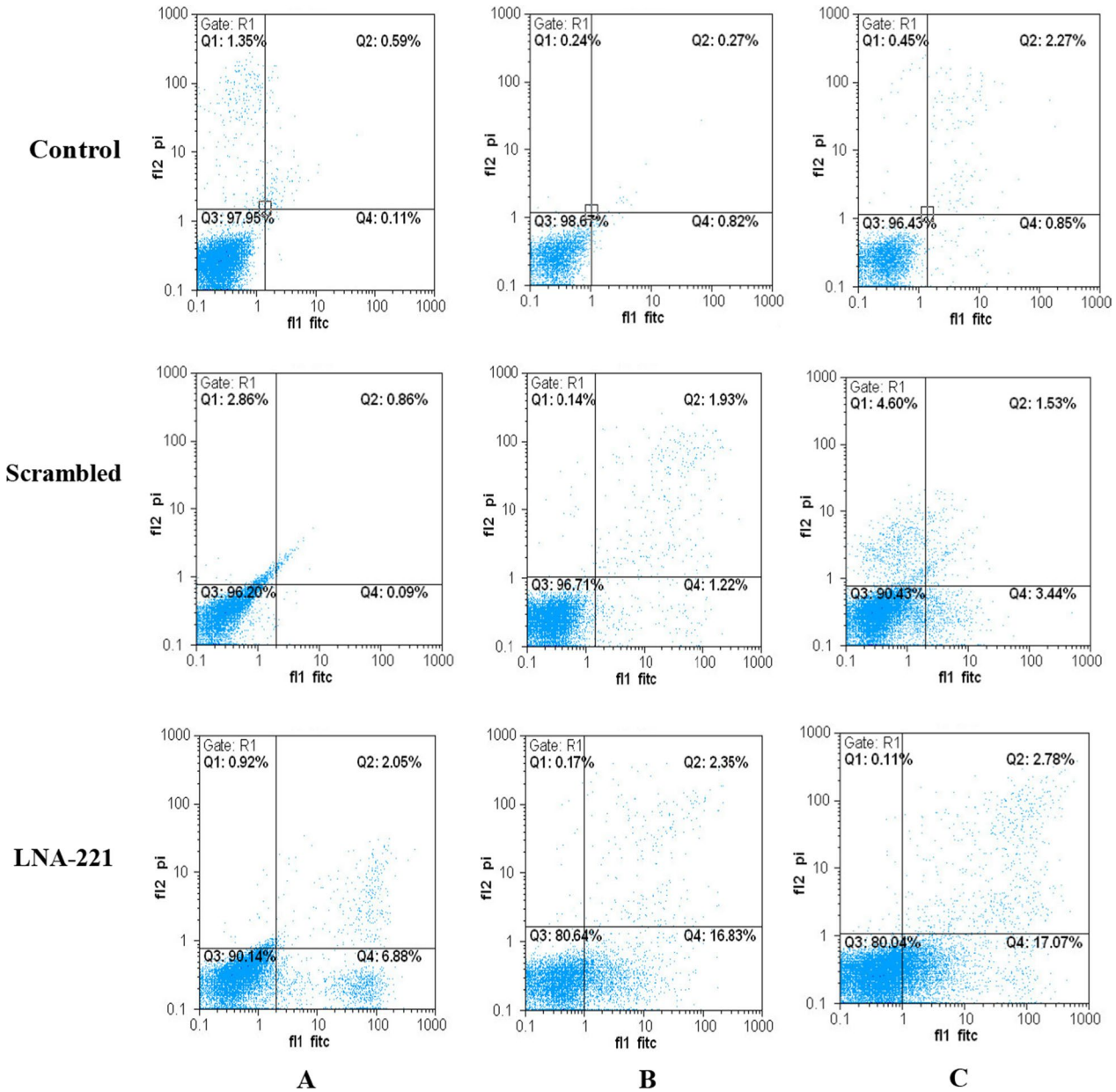

Fig. 2 Evaluation of apoptosis in untreated cells, scrambled- LNA, and LNA-Anti-miR groups by Annexin V-PI staining. This assessment was performed at 24, 48, and 72 h after transfection (**A**, **B**, **C**). Flow cytometry analysis was performed by using 500 nm excitation and 520 nm bandpass filter for the fluorescein detection

breast cancer (BC) [23], hepatocellular carcinoma (HCC) [24], and lung cancer [25].

Various studies have further revealed that silencing oncogenic miRNAs can be a successful strategy to treat cancer in humans. In fact, the disruption of a specific molecule (viz. miR) is of importance in cancer development [26, 27]. Against this background, this study aimed to employ the engineered nucleic acid, such as LNA, as a new strategy to target miRNA and resist against nucleases. Moreover, LNA-Anti-miR was applied for the miR-221 inhibition in the 183-E95 B-CLL cells. Although the C582 cells were used, no significant data were obtained for the miR-221 alternation. Thus, the C582 results were excluded. Preventing the cell proliferation after the LNA transfection could further efficiently exhibit the miR-221 cell line inhibition. The gene expression data additionally showed a decrease in the miRNA expression levels following the LNA transfection. The

reduction in the miR-221 inhibition also resulted in cell viability loss in the MTT assay. Correspondingly, the apoptosis assay supported these results, and a significant increase in the apoptosis was observed by the LNA-anti-miR-221 in 183-E95 cells. Besides, there was the lowest decline in cell viability in the scrambled LNA group, and no significant difference was found between the untreated cells and other groups. Further evidence on oncomiR indicated that the therapeutic approach concerned had been used for many types of cancer. Actually, the LNA-anti-miR-221 inhibited the mutated cells, CLL p53-mutant MEG-01, and thus significantly elevated caspase activity in the cell line under Fludarabine as a chemotherapy drug [28]. The increased levels of miR-221/222 in the MEC1 cells also reduced p27 protein. Thus, p27 and miR-221/222 created a CLL cell-stabilizing regulatory loop in a resting state [29].

Other studies, showing the inhibitory impact of miR-221/222 on multiple myeloma cancer cells in vitro, had similarly demonstrated that the LNA technology could be applied to inhibit miR-221/222. Such investigations had shed light on the miR-221/222 inhibition in some myeloma malignant tumor cells, which could cause an increase in PTEN and PUMA genes and $p27^{Kip1}$, and contribute to inhibiting the tumors in cell cycles and decreasing cell proliferation [18]. In Frenquelli et al., the expression levels of miR-221/miR-222 and p27 in peripheral blood (PB) leukemia cells from 38 patients with CLL had been inversely correlated. In addition, when CLL cells had been induced in vitro with cytosine-phosphate-guanine oligodeoxynucleotides (CpG-ODNs) to enter the cell cycle, a significant growth had been evident in the miR-221/222 expression, and there was a marked downregulation of the p27 protein. These data indicated that the miR-221/222 cluster could modulate the p27 protein expression in the CLL cells, and suggested that miR-221/222 and p27 might represent a regulatory loop, contributing to the CLL cells in a quiescent state [29]. In Ashrafi et al., the effective inhibition of miR-222 in the B-CLL cell viability was evaluated. In this study, the miR-222 inhibition was performed in the B-CLL cell line (183-E95), using the LNA antagomir. The miR-222 downregulation in the B-CLL cell line (183-E95) with the LNA antagomir had further reduced cell viability in B-CLL. Based on these findings, the miR-222 inhibition could represent a potentially new approach to treat B-CLL [14].

According to Gimenes-Teixeira, miR-221 could be a useful biomarker in T-cell acute lymphoblastic leukemia (T-ALL). Accordingly, the patients with T-ALL/CD56 with the high expression levels of miR-221, and those with T-ALL/CD56+, had accordingly shown a shorter oxidative stress; therefore, they required more careful monitoring of treatment response and/or could be considered as candidates for more intensive therapy [30]. In another study by Jong-Kook et al., the miR-21 and miR-221 inhibition had increased apoptosis in the HS766T cells by three- to sixfold compared to the control oligonucleotide [31]. In Galardi et al., miR-221 and miR-222 had been further over-expressed in the PC3 cellular model of aggressive prostate carcinoma, as compared with the LNCaP and 22Rv1 cell line models of slow-growing carcinoma. The results had further suggested that the miR-221 and miR-222 over-expression might contribute to prostate cancer growth and progression, at least in part by blocking the p27 mRNA translation [16].

Further investigations have also illustrated that the liver cancerous cell proliferation has been stopped using the miR-221 inhibitors, modified with LNA. Likewise, in vitro miR-221/222 over-expression can target a DNA damage-inducible transcript 4 (DDIT4) as a mammalian target of rapamycin (mTOR) pathway modulator [19]. In the status quo situation, chemotherapy is thus a common approach to treat CLL. In some cases, patients acquire resistance to this treatment, while, using novel approaches such as inhibition of oncomirs, which causes cell susceptibility to chemotherapy, makes the therapeutic strategies much more effective.

Conclusion

It was concluded that the inhibition of the miR-221 expression by LNA-anti-miR was an approach to treat B-CLL, which could be actually employed individually or in combination with the existing therapies in the reduction of the current restrictions to treat such malignancies. Despite the convincing results, this approach should be applied in in vivo or animal models to confirm its effectiveness.

Abbreviations

B-CLL: B-chronic lymphocytic leukemia; Cdk: Cyclin-dependent kinase; LNA: Locked nucleic acid; RT-qPCR: Reverse transcription-quantitative polymerase chain reaction; FC: Flow cytometry; ANOVA: Analysis of variance; miRNA: MicroRNA; 3′-UTR: 3′-Untranslated region; Let-7: Lethal-7; ZAP-70: Zeta-associated protein of 70; IgHV: Immunoglobulin heavy-chain variable; GBM: Glioblastoma; TPC: Thyroid papillary carcinoma; RPMI: Roswell Park Memorial Institute; FCS: Fetal calf serum; CO_2: Carbon dioxide; hsa: Homo sapiens, human; 6-FAM: 6-Carboxyfluorescein; FM: Fluorescence microscopy; cDNA: Complementary DNA; DMSO: Dimethyl sulfoxide; PI: Propidium iodide; HCC: Hepatocellular carcinoma; BC: Breast cancer; PB: Peripheral blood; T-ALL: T-cell acute lymphoblastic leukemia; DDIT4: DNA damage-inducible transcript 4; mTOR: Mammalian target of rapamycin.

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Author contributions

This work was conceived and designed by MMS, KAD. The experiments were carried out by MAS. The manuscript was prepared by KAD, MMS, ASH. All authors read and approved the final manuscript.

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Author details
[1]Medical Plants Research Center, Basic Health Sciences Institute, Shahrekord University of Medical Sciences, Shahrekord, Iran. [2]Research Center for Molecular Medicine, Hamadan University of Medical Sciences, Hamadan, Iran. [3]Thalassemia and Hemoglobinopathy Research Center, Health Research Institute, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran. [4]Cellular and Molecular Research Center, Basic Health Sciences Institute, Shahrekord University of Medical Sciences, Shahrekord, Iran.

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