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Alterations in The Plasma Expression of mir-15b, mir-195 and the Tumor-Suppressor Gene DLEU7 in Patients with B-Cell Chronic Lymphocytic Leukemia

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Abstract

Background: Chronic lymphocytic leukemia (CLL) is one of the most prevalent forms of leukemia in adults. Inactivation of the DLEU7 gene is frequently observed in patients with CLL. Furthermore, microRNAs (miRNAs) have been observed to have a critical role in the pathogenesis of several cancers, including leukemia. Considering the tumor-suppressive role of DLEU7, as well as the tumor suppressor or oncogenic role of microRNAs (miRNAs), the aim of the present study was to evaluate the potential miRNAs targeting the DLEU7 gene in B-cells and explore expression changes these genes in the plasma of B-CLL patients.

Methods: The miRNAs interacting with the DLEU7 gene were predicted and selected using bioinformatics tools. A total of 80 plasma samples were collected from 40 patients with B-cells and 40 healthy individuals, then subjected to RNA extraction and cDNA synthesis. The expression profiles of the predicted miRNAs and the DLEU7 gene in the plasma of B-CLL patients and healthy individuals were determined by RT-qPCR analysis.

Results: The bioinformatics prediction indicated that miR-15b and miR-195 target the DLEU7 gene. The expression levels of miR-15b and miR-195 were significantly higher in the plasma of patients with B-CLL compared to the healthy individuals (91.6, p= 0.001) (169, p= 0.001). However, the expression level of the DLEU7 gene was found to be significantly lower in the patient group compared to healthy controls (0.304, p= 0.001).

Conclusions: Both miR-15b and miR-195, have the potential to function as novel and non-invasive biomarkers in the diagnosis and prognosis of patients with B-CLL.

Keywords: B-CLL, miRNA, Biomarker, DLEU7, RT-QPCR.

Introduction

Chronic lymphocytic leukemia (CLL) is one of the most common forms of leukemia, primarily affecting the older adult population (1). This subtype accounts for about 25% to 30% of all leukemia cases (2). CLL is a chronic lymphoproliferative disorder characterized by the proliferation of malignant and dysfunctional B lymphocytes that appear mature. These abnormal B-cells accumulate in the blood, bone marrow, and secondary lymphoid tissues (3). On their cell surface, they express CD5, CD19, CD23, and low levels of membrane immunoglobulins (IgM and IgD) (4). Therefore, they have a distinct phenotypic profile

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distinguishing them from normal B cells (5). It has been shown that one of the important prognostic factors in CLL are chromosome abnormalities, including the deletion of chromosomes 17q13, 6q21, 11q23, 13q14 and 17p13, and trisomy 12 (6, 7). Deletions of 13q14 are the most prevalent chromosomal abnormalities observed in CLL, occurring in over 50% of CLL patients (8, 9). Research has shown both miR15/5 and DLEU7 (deletion in lymphocytic leukemia 7) comprise the minimum deleted region (MDR) in CLL (10, 11).

DLEU7 is known as a tumor-suppressor gene at 13q14 (11). This molecule acts as an inhibitor of the NF-kB signaling pathway, blocking the function of TACI (transmembrane activator and calcium modulator and cyclophilin ligand interactor) and BCMA (B-cell maturation antigen) through TRAFs (Tumor necrosis factor receptor-associated factors) (12) (Fig. 1A). Deletion of 13q14 in CLL leads to the inactivation of DLEU7 and consequently the induction of the NF-kB signaling pathway through TRAFs (13) (Fig. 1B). The NF-kB signaling pathway has a significant role in the pathogenesis of CLL leading to proliferation, survival and the inhibition of apoptosis in CLL cells (14).

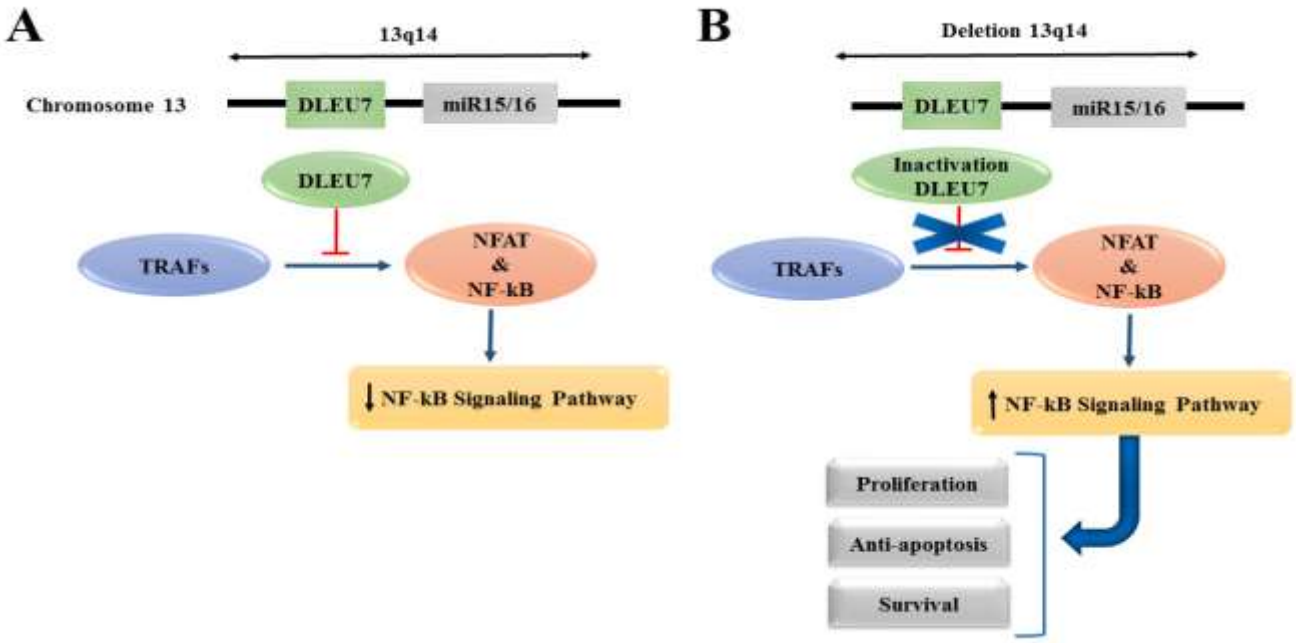

Fig. 1. Schematic representation of the role of the DLEU7 molecule in the NF-kB signaling pathway. A) DLEU7 inhibits NF-kB signaling pathway through TRAFs. B) Inactivation of DLEU7 induces the NF-kB signaling pathway through TRAFs in CLL cell.

MicroRNAs (miRNAs) as a type of short non-coding RNAs play an important role in the regulation of gene expression via binding to the 3′ untranslated region (3′ UTR) of target mRNAs and inhibiting translation (15, 16). These miRNAs have been observed to have a role in a range of different cellular and molecular processes including proliferation, differentiation, metabolism, apoptosis and tumorigenesis (17, 18). These molecules are found in different tissues with variable expression, and their expression profiles become alter in a disease state (19-21). Additionally, the miRNAs are stable in serum and plasma and have unique expression profiles in different forms of cancer (22-24). Therefore, these molecules are promising candidates as non-invasive biomarkers in the diagnosis and treatment of various diseases, including CLL (25, 26).

In the present study, miRNAs targeting the DLEU7 gene were predicted using bioinformatics tools. The expression levels of the predicted miRNAs and DLEU7 in the plasma samples of CLL patients was then determined using RT-qPCR.

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Materials and Methods

Clinical samples

A total of 80 plasma specimens were collected. Of the samples, 40 were isolated from patients diagnosed with B-CLL and 40 were from healthy volunteers. In both groups, samples were collected from 20 males and 20 females, with an average age of 35 years (ranging from 30 to 67 years) in the patient group and 34 years (ranging from 26 to 68 years) in the healthy control group (Table 1). Participants in the B-CLL group had not receive any prior conventional treatment including surgery, chemotherapy, or radiotherapy. Prior to taking part in this study, all participants provided written informed consent.

Prior to analysis, all collected samples were stored at -70 ˚C. Plasma was then extracted from all peripheral blood samples. After collecting the samples, flow cytometry was used to confirm the diagnosis of B-CLL. Flow cytometry specific markers CD5+, CD19+, CD20+ and CD23+ were used to identify B-CLL. In this group we did not express any aberrant markers such as those specific to myeloid cells or T-lymphocytes. This study was approved by the Ethical Committee of Arak University of Medical Science, Ethic Approval Code IR.ARAKMU.REC.1395.418.

Available	Total	Sex		Average age (range)	Hematology parameters in peripheral blood		
		Female	male		WBC 109/L	Hb gr/L	Plt 109/L
B-CLL patients Number (%)	40 (50)	20 (50)	20 (50)	35 (30-67)	54.2	10.2	112
Healthy controls Number (%)	40 (50)	20 (50)	20 (50)	34 (26-68)	5.2	14.7	281

Table 1. Baseline characteristics of the B-CLL patients and control group.

Bioinformatics analysis

Determining the miRNAs that interact with the DLEU7 gene were predicted using bioinformatics websites such as PicTar (https://pictar.mdc-berlin.de/), DIANA (http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=microT_CDS/index), Targetscan (http://www.TargetScan), miRDB (http://mirdb.org/), miRecords (http://c1.accurascience.com/miRecords/) and RNAhybrid (https://bio.tools/rnahybrid). Based on the results obtained from different bioinformatics tools, two miRNAs with the highest scores and repetition were selected and used for further *in vitro* investigation. The sequences of the predicted miRNAs were retrieved from the miRBase (www.mirbase.org) database.

Extraction of miRNA and reverse transcription

The extraction of miRNAs from plasma samples was performed using the RNX-Plus kit (SinaClon, Iran) according to the manufacturer`s protocol. The extracted miRNAs were converted to cDNA using a mixture containing 1μM of specific stem-loop RT primers (Table 2), M-MLV enzyme (as reverse transcription enzyme) (Vivantis, Malaysia), 1x RT-enzyme buffer, 1 μg of RNA, and 400 μM dNTP. The mixture was first incubated at 75 °C for 5 min and then placed in a thermal cycler (Eppendorf, Germany) at 25 °C for 15 min, 37 °C for 15 min, 42 °C for 45 min, and then 75 °C for 10 min. The resultant synthesized cDNAs were stored at -20 °C.

Quantitative real-time PCR

QRT-PCR was performed to detect the expression level of miRNAs and the DLEU7 gene in the plasma samples of patients with B-CLL using SYBR Green PremixExRaq II (Yekta Tajhiz Azma, Iran) and the Light Cycler96 instrument (Roche, Germany). The comparative Cq (quantitation cycle) method was applied to quantify the expression levels using the relative expression software tool (REST) (27). The specific primers for qRT-PCR were designed by the GeneRunner and AlleleID7 software (Table 3) and the specificity of primers was determined using the NCBI BLASTn tool. As reference genes for qRT-PCR, miR-103 and GAPDH were used as an endogenous normalizer miRNA and as a housekeeping gene, respectively.

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Table 2. List of stem-loop primers used for C-DNA synthesis.

Target	Primer sequences (5`-3`)
miR-15b	5`-GTCGTATCGAGAGCAGGGTCCGAGGTATTCGCACTCGATACGACTGTAAAC-`3
miR-195	5`-GTCGTATCGAGAGCAGGGTCCGAGGTATTCGCACTCGATACGACGCCAATA-`3
miR-103	5`-GTCGTATCGAGAGCAGGGTCCGAGGTATTCGCACTCGATACGACCAAGGCA-3`

Table 3. List of specific primers used for qRT-PCR.

Target	Primer sequences (5`-3`)
miR-15b	Forward: 5`-GCAGCACATCATGGTTTACA-`3 Reverse: 5`-AGAGCAGGGTCCGAGGT-3`
miR-195	Forward: 5`-GCAGCACAGAAATATTGGC-`3 Reverse: 5`-AGAGCAGGGTCCGAGGT-3`
miR-103	Forward: 5`-GCTTCTTTACAGTGCTGCC-3` Reverse: 5`-AGAGCAGGGTCCGAGGT-3`
DLEU7	Forward: 5`-CCATTCACCTGAAGGATAGTG-`3 Reverse: 5`-TTAGCAAGTGACTGAATCAGC-`3
GAPDH	Forward: 5`-GGAGTCCACTGGCGTCTTCAC-3` Reverse: 5`-GAGGCATTGCTGATGATCTTGAGG-3`

Data analysis

The relative expression rate from qRT-PCR was analyzed using REST (2009) and determined as the mean±standard error (SE). All results were analyzed using the statistical package for social sciences (SPSS) software (Ver 16; SSPS Inc., 184 Chicago). Significant differences were calculated and considered statistically significant at p values< 0.05.

Results

MiRNAs prediction

The predictions of various bioinformatics databases such as PicTar, DIANA, Targetscan, miRDB, miRecords, and RNAhybrid revealed that miR-15b and miR-195 are the miRNAs that target the DLEU7 gene with the high repetition (Table 4). Therefore, these two miRNAs with highest scores were chosen as the miRNAs that target the DLEU7 mRNA. Some of the databases, including Pictar, DIANA, Targetscan, and miRDB, reported the connection of miR-15b and miR-195 to the DLEU7 gene based on the indicators, free energy, miTG score, context score, and target score, respectively (Table 5), while miRecords and RNAhybrid only revealed connection or non-connection. The 3′-UTRs region of DLEU7 mRNA that is targeted by the miR-15b and miR-195 binding seed regions were shown in PicTar (Fig. 2A) and Targetscan softwares (Fig. 2B).

Table 4. The results of miRNA prediction for DLEU7 gene

miRNname	Targetscan	PicTar	DIANA	miRecords	miRDB	RNAhybrid	SUM
Has-miR-15b	1†	1	1	1	1	1	6/6 §
Has-miR-195	1	1	1	1	0‡	1	5/6

†: Targeting of DLEU7 is confirmed by the software (Targetscan, PicTar, DIANA, miRecords, miRDB and RNAhybrid)
‡: Targeting of DLEU7 is not confirmed by the software.
§: Number of repetitions / total number of databases reviewed.

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Table 5. Scores obtained from different bioinformatics databases for the selected miRNA.

miRNA	Score			
	PicTar (Free Energies kcal/mol)	DIANA (miTG score) †	Targetscan (context score) ‡	miRDB (Target score) §
Has-miR-15b	-20.6	0.996	-0.39	81
Has-miR-195	-21.1	0.994	-0.38	81

† MiRNA target gene (miTG score): The prediction score. The higher the miTG score the higher the probability of targeting.
‡ Context score (CS): The context score is the sum of the contribution of site-type contribution, 3' pairing contribution, local AU contribution and position contribution features.
§ Target score: The higher the score, the more confidence we have in this prediction. Predicted target with prediction score > 80 is most likely to be real. If the score is below 60, you need to be cautious, and it is recommended to have other supporting evidence as well.

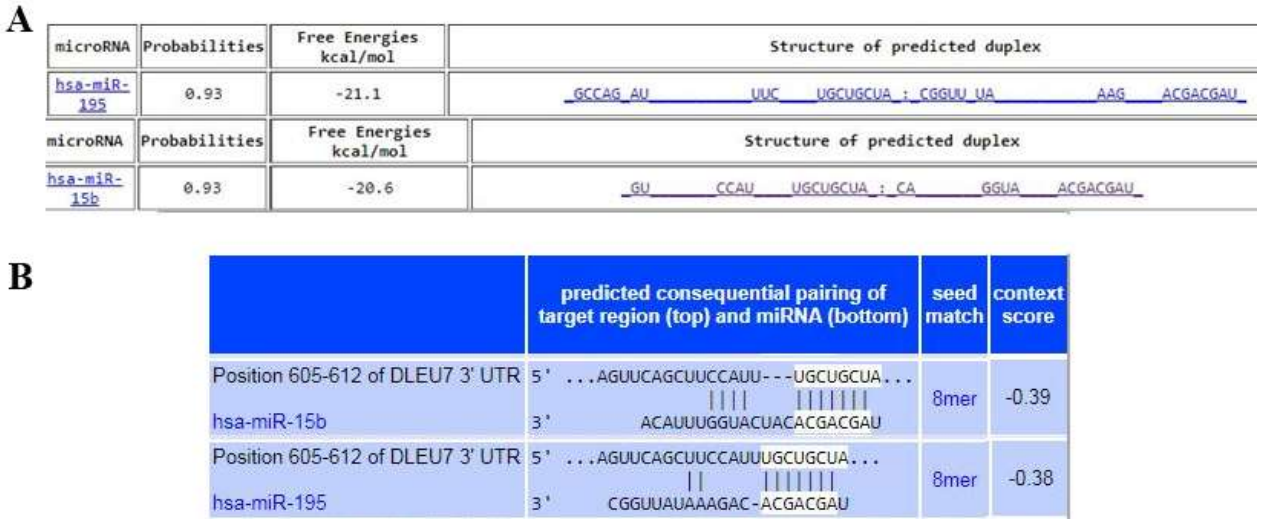

Fig. 2. Schematic representation of the 3′-UTRs region of DLEU7 mRNA that is targeted by miR-15b and miR-195 binding seed region in A) PicTar and B) Targetscan software.

Purification of PIA

The predictions of various bioinformatics databases such as PicTar, DIANA, Targetscan, miRDB, miRecords, and RNAhybrid revealed that miR-15b and miR-195 are the miRNAs that target the DLEU7 gene with the high repetition (Table 4). Therefore, these two miRNAs with highest scores were chosen as the miRNAs that target the DLEU7 mRNA. Some of the databases, including Pictar, DIANA, Targetscan, and miRDB, reported the connection of miR-15b and miR-195 to the DLEU7 gene based on the indicators, free energy, miTG score, context score, and target score, respectively (Table 5), while miRecords and RNAhybrid only revealed connection or non-connection. The 3′-UTRs region of DLEU7 mRNA that is targeted by the miR-15b and miR-195 binding seed regions were shown in PicTar (Fig. 2A) and Targetscan software (Fig. 2B).

Determination of the expression level of miR-15b, miR-195 and DLEU7 gene

The results obtained from qRT-PCR demonstrated that the expression levels of the DLEU7 gene were significantly lower in B-CLL patients than in healthy individuals (0.304, $p< 0.001$) (Fig. 3A). However, the expression levels of miR-15b and miR-195 were significantly higher in B-CLL patients

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than in the healthy controls (91.6, p< 0.001) (169, p< 0.001) (Fig. 3B). Therefore, the expression levels of miR-15b and miR-195 were increased while the expression level of the target gene of these two miRNAs, DLEU7, was decreased in B-CLL (Fig. 4).

Since the deletion of 13q14 is found in patients with B-CLL, the expression of the DLEU7 gene may be decreased (50% of cases) as a result of this deletion (8). In another group of B-CLL patients without the 13q14 deletion, the expression of the DLEU7 gene and miR-15b did not change. The difference between these two groups can be seen in the distribution of ΔCt in the expression of DLEU7 gene (Fig. 5A) and even miR-15b (Fig. 5B).

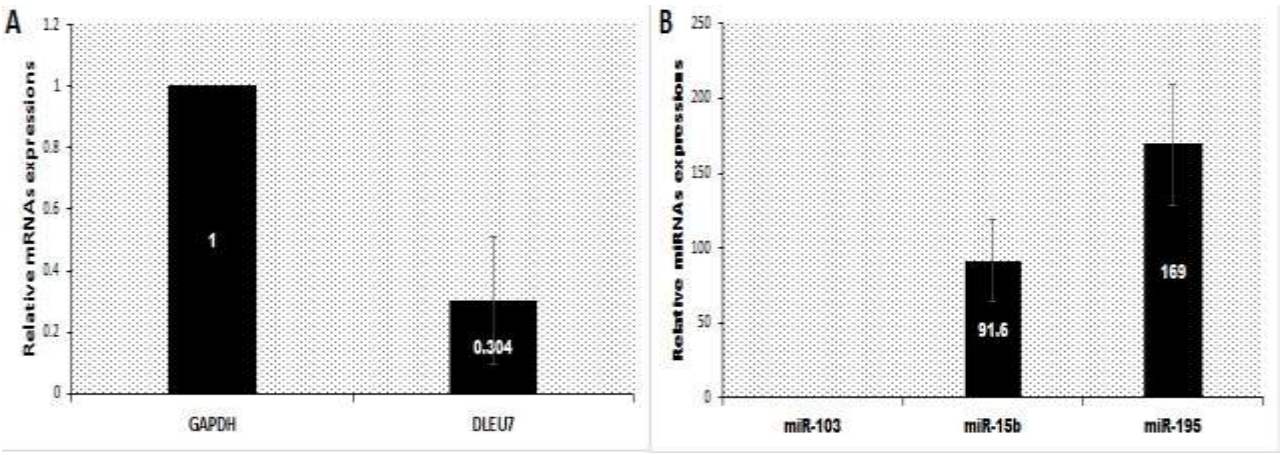

Fig. 3. (A) Relative expression of DLEU7 in B-CLL patients in comparison to the healthy control group. (B) Comparison of differential expression levels of miR-15b and miR-195 between patients with B-CLL and healthy individuals. Error bars indicate the standard error of the mean.

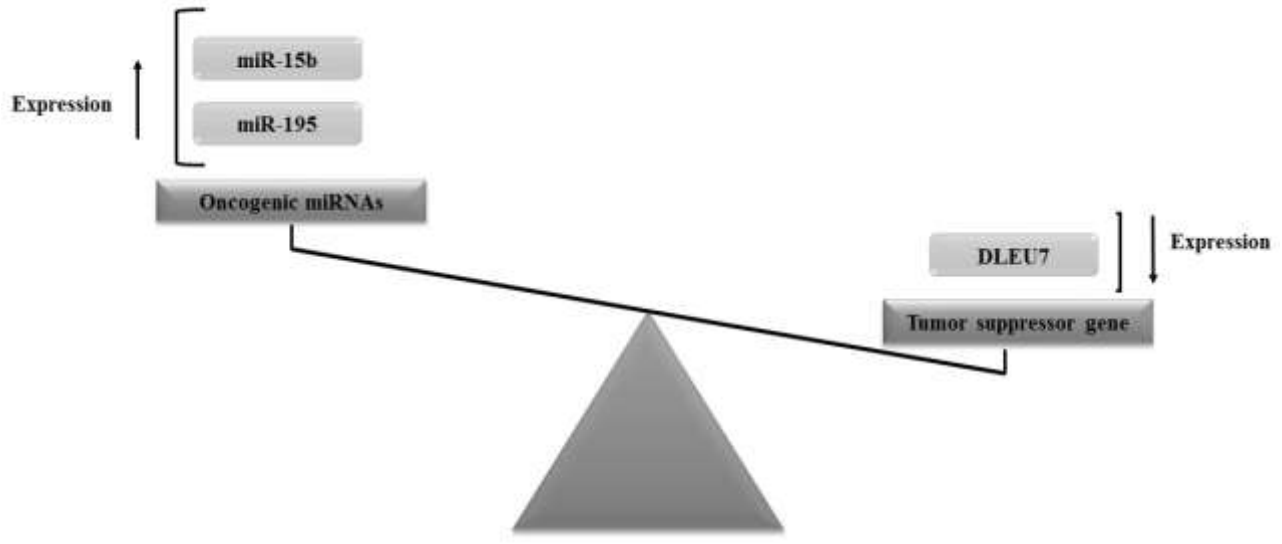

Fig. 4. Schematic representation of the increased expression of oncogenic miRNAs such as miR-15b and miR-195 and decreased expression of the DLEU7 suppressor tumor gene (as the target gene for these miRNAs) in B-CLL.

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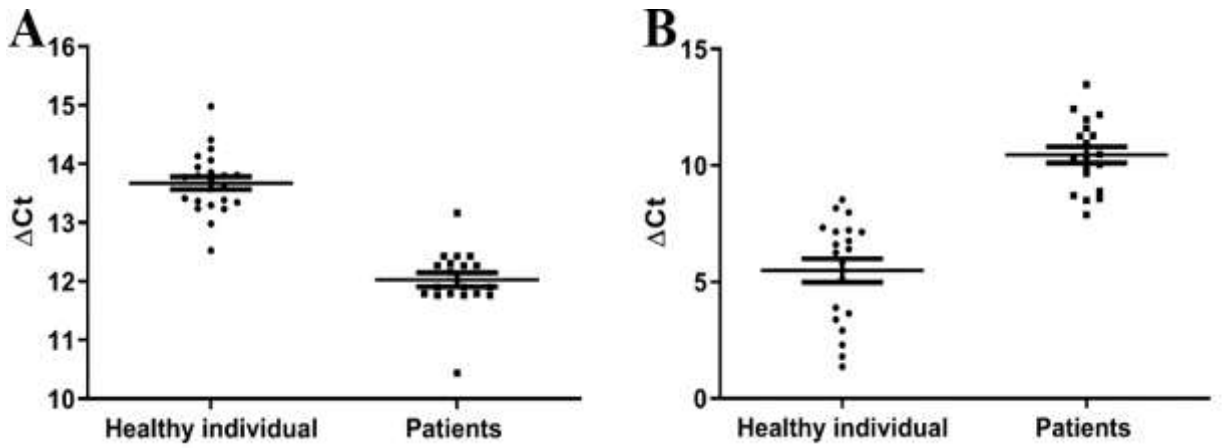

Fig. 5. Distribution of ΔCt in healthy individuals and patients with B-CLL for (A) DLEU7 and (B) miR-15b.

Discussion

Genetic alterations such as mutations, chromosomal abnormalities, epigenetic modifications and alterations in the expression of miRNAs are one of the most valuable prognostic factors in CLL (28). Evidence has demonstrated that the dysregulation of miRNAs is associated with the development and progression of CLL (29, 30). These miRNAs can target different molecules that play an important role in the pathogenesis of CLL (31). The expression of miRNAs in many disease states and their unique properties such as tissue specificity, rapid release rate, and plasma stability make them promising diagnostic and therapeutic biomarkers in many diseases, including CLL (32). Several reports have indicated that different miRNAs including miR-15, miR-16, miR-17, miR-21, miR-155, miR-192 and miR-181 are involved in CLL pathogenesis (19, 33, 34). It has been shown that some miRNAs including miR-34a, miR-155, and miR-342-3p have been found to be significantly up-regulated in CLL patients, whereas miR-103, miR-181a and miR-181b are down-regulated (35).

Some studies have indicated that DLEU7, as a tumor suppressor gene at 13q14, downregulates the NF-kB signaling pathway and that dysregulation of this signaling pathway contributes to development and progression of CLL (12). Therefore, given the potential role for DLEU7 in the pathogenesis of CLL, this gene appears to be a suitable candidate for miRNA targeting studies.

In the present study, we predicted miRNAs that interacted with the DLEU7 gene using bioinformatics tools. We then measured the expression levels of these predicted miRNAs and DLEU7 in the plasma samples of CLL patients and healthy controls using RT-qPCR. Based on our bioinformatics study, miR-15b and miR-195 had the highest scores and were therefore selected for further experiments. Our findings demonstrated that these two miRNAs likely target the DLEU7 gene.

The plasma levels of miR-15b and miR-195 were found to be significantly higher in patients with CLL than in the healthy individuals. The expression of miR-15b and miR-195 was upregulated by 91.6-fold (p= 0.001) and 196-fold (p= 0.001), respectively. However, the plasma levels of DLEU7 were downregulated by 0.304 (or −3.28-fold, p= 0.001) in the CLL patients compared to healthy individuals. These results suggest that miR-15b and miR-195 likely function as oncogenes in CLL and contribute to the proliferation, survival, and inhibition of apoptosis of the malignant CLL B-cells by directly targeting DLEU7 mRNA.

The role of miR-15b expression has been extensively studied in different types of cancer including hepatocellular carcinoma, breast cancer, gastric cancer, and glioma by targeting E2F, MTSS1, BCL-2, NRP-2, and cyclin D1, respectively (36-39). Therefore, the abnormal expression of miR-15b found in various cancers makes these miRNAs an effective molecular

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biomarker (40). Interestingly, miR-195 has been found to be both upregulated and deregulated depending on the type of cancer. For example, miR-195 has been found to be upregulated in breast cancer and CLL, while downregulated in adrenocortical carcinoma and hepatocellular carcinoma (32, 41-43). Bioinformatics predictions have also indicated that miR-195 can target several molecules such as BCL2, IGF1, cyclin D3 and surviving in colorectal cancer, rectal cancer, and lung cancer, respectively (44-46).

To the best of our knowledge, this study is the first report to examine DLEU7-related miRNAs in CLL. The findings of the present study indicate that there is an inverse relationship between the upregulation of the miRNAs, miR-15b and miR-195, and the downregulation of DLEU7 in CLL patients. This relationship may be due to the targeting of the DLEU7 mRNA by miR-15b and miR-195. Therefore, we propose that these miRNAs have the potential to function as novel and non-invasive biomarkers in the diagnosis and prognosis of CLL. However, further investigation is needed to uncover the functional relationship between the identified miRNAs and the DLEU7 gene.

Further research, including Luciferase assay and FISH, is needed to confirm that the decrease in the expression of the DLEU7 mRNA is due to the aberrant expression of these miRNAs, not to the deletion of del13.

Acknowledgements

The study was approved by the Ethics Committee of Arak University of Medical Sciences, in accordance with the declaration of Helsinki, (ethics number IR.ARAKMU.REC.1395.418).

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