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Research Article

Down-regulation of *miR-193b-3p* and *miR-376a-3p* in Chronic Lymphocytic Leukemia

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Abstract

Background: Chronic lymphocytic leukemia (CLL) is the most common adult human leukemia. Studies revealed that microRNAs (miRNAs) can function as oncogenes or tumor suppressors in CLL and that the expression of miRNAs, such as *miR-193b-3p* and *miR-376a-3p* change in several diseases. We aimed to elucidate the changes in miR-193b-3p and miR-376a-3p expression in CLL and determine their potential as diagnostic biomarkers for this disease.

Materials and Methods: We investigated *miR-193b-3p* and *miR-376a-3p* expression by quantitative real-time PCR in peripheral blood mononuclear cells of 30 patients with CLL and 30 healthy individuals. Moreover, *in silico* molecular enrichment analysis was conducted on predicted and validated targets of *miR-193b-3p* and *miR-376a-3p* from the miRecords and miRTarBase databases.

Results: The expression of *miR-193b-3p* and *miR-376a-3p* was significantly different between the two groups ($P<0.0001$ and $P < 0.0001$, respectively).

Conclusion: Based on these findings, *miR-193b-3p* and *miR-376a-3p* could be novel biomarkers for the early diagnosis of CLL and could be used to design new CLL control strategies.

Keywords: chronic lymphocytic leukemia, *miR-193b-3p*, *miR-376a-3p*, miRNA

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1. Introduction

Chronic lymphocytic leukemia (CLL) is a clonal proliferative tumor of mature B lymphocytes characterized by the lymphocyte accumulation in the peripheral blood, bone marrow, and lymph nodes [1]. CLL is characterized by asymmetrical proliferation and apoptosis of leukemia cells along with expressing the CD5 and CD19 markers. Several chromosomal abnormalities were detected as the most common abnormalities in CLL, including deletions at 13q, 11q, 17p; and trisomy 12 [2, 3]. This leukemia is the most common type of leukemia in the western world, causing ~5,000 mortalities in the United States annually [4]. Most patients with CLL are able to survive for several years and show mild symptoms [5]. One of the important factors to be considered in cancer therapy, which helps to plan and choose better treatment options, is the early diagnosis. Discovery of biomarkers at different levels, including genomics, transcriptomics, and proteomics levels can help to better diagnose and treat several cancers, including CLL [6]. Unfortunately, the precise molecular mechanisms underlying the CLL progress still unclear, which limit early diagnosis and timely treatment. Thus, tracking the molecular mechanisms of CLL progression and its biomarkers to improve the likelihood of early diagnosis have become the focus of CLL research. Some evidence suggests that multiple genes, miRNAs, and cellular pathways are involved in the incidence and progression of CLL [7, 8]. MicroRNAs (miRNAs) are short non-coding regulatory RNAs that control the gene expression at the post-transcriptional level by binding to the 3′-untranslated region (UTR) or 5´UTR of their mRNA targets, resulting in inhibition or degradation of mRNA. miRNAs play a significant role in cancer progression by acting either as oncogenes or tumor suppressors [9]. miRNAs expression profiles have already entered cancer clinics as diagnostic biomarkers to recognize tumor initiation, progression, and response to treatment in patients with cancer [10]. The first human disease-related miRNAs, *miR-15* and *miR-16* at 13q14, were characterized in CLL [11, 12]. Some evidences indicated that deregulation of miRNAs could be associated with CLL [13]. Moreover, some studies have shown that the expression of *miR-193b-3p* is altered in several biological samples of cancer patients, such as acute lymphoblastic leukemia (ALL), prostate cancer, and ovarian cancer [14–16]. Additionally, changes in the expression of *miR-376a-3p* have been associated with various human diseases, such as ovarian cancer and hepatocellular tumor of bone [15–17]. However, the roles of *miR-193b-3p* and *miR-376a-3p* in CLL disease has remained unexplored, till date. In this study, the expression of *miR-193b-3p* and *miR-376a-3p* was examined by quantitative real-time PCR in peripheral blood mononuclear cells (PBMCs) of patients with CLL and healthy individuals. Therefore, the aim of our study was to

determine whether the deregulated expression of *miR-193b-3p* and *miR-376a-3p* (as these two miRNAs are involved in CLL) can be used as a new diagnostic biomarker in patients with CLL.

2. Materials and Methods

2.1. Patients and controls

Blood samples were collected from patients with CLL and from randomly selected healthy individuals (both male and female). Totally, 60 samples, including 30 from patients with CLL [diagnosed in the Omid Hospital (Isfahan, Iran)] and 30 from healthy individuals, were selected for the study. CLL was diagnosed based on blood cell count and cell morphology. The exclusion criteria were as follows: (i) CLL diagnosis more than 12 months before registration; (ii) Clinical Binet stage B or stage C; (iii) need for therapy according to National Cancer Institute (NCI) guidelines [19]. Six milliliters of peripheral blood was collected in EDTA-containing tubes and immediately transported on ice to the laboratory. The Ethics Committee of the Omid Hospital (Isfahan, Iran) approved the protocol of this study. Written informed consent was obtained from all participating individuals prior to sample collection.

2.2. Complete blood count

Complete blood count (CBC) test was assessed using CA&XN-Series TM Automated Hematology Analyses (Kobe, Japan). The Sysmex XN series uses fluorescence flow cytometry. Some variables associated with CLL were determined by this device.

2.3. Peripheral blood mononuclear cell isolation from blood

Peripheral blood mononuclear cells (PBMCs) were isolated from blood samples by density gradient lymphoprep (Bio Sera, Kansas City, USA) based on the manufacturer's protocol. Mononuclear cells, monocytes, and lymphocytes have lower densities in comparison with erythrocytes and leukocytes; therefore, after centrifugation, they remain in an intermediate phase. Briefly, 4 ml of blood was diluted at a ratio of 1:1 with physiological saline and gradually added to the 4 ml lymphoprep solution gradient in a Falcon tube. The tubes were centrifuged at 800 ×*g* for 30 min, and then PBMCs were transferred

from the middle phase into 2-mL RNAase-free microtubes and frozen at −70 ˚C until next step.

2.4. MiRNA extraction

MiRNA was extracted from PBMCs using miRNA Hybrid-R (Geneall, Seoul, Korea) based on the manufacturer's instructions. Quality of the extracted miRNA was determined according to the 260/280 absorbance ratio, measured by NanoDrop spectrometer (Thermo Scientific, Waltham, MA, USA).

2.5. Complementary DNA synthesis and real-time polymerase chain reaction

Complementary DNA (cDNA) synthesis for *miR-193b-3p* and *miR-376a-3p* was carried out using a universal cDNA synthesis kit (Exiqon, Denmark) using poly-A tailing, according to manufacturer's instructions. Real-time quantitative PCR reactions were carried out in triplicate by using standard protocols with an ABI PRISM 7500 instrument (Applied Biosystems, USA). Briefly, in a total volume of 10 µl, 20 ng/µl of cDNAs were added to a master mix comprising 10 pmol/µl of each *miR-193b-3p* or *miR-376a-3p* primers (Exeqon, Denmark) and 5 ml of SYBR premix ExTaq II (TaKaRa, Kusatsu, Shiga Prefecture, Japan). The program for the run was set as follows: 95 ˚C for 15 min followed by 40 cycles of 95 ˚C for 15 s, 60 ˚C for 30 s, and 72 ˚C for 30 s. The PCR reaction was followed by a melting curve program (60–75 ˚C with a temperature transition rate of 1 ˚C s^{-1} and a continuous fluorescence reading). Real-time PCR data analysis was performed using the ΔΔCT method[20] and the final data were normalized using small nuclear RNA, *U6*, expression level as an endogenous control.

2.6. Statistical analysis

For the statistical study, Graph Pad Prism statistical software, version 5.01 (Graph Pad, San Diego, CA, USA) was used. Normality was evaluated by the Kolmogorov–Smirnov test. The independent samples *t*-test was applied to analyze the data between groups. For all the tests, $p \leq 0.05$ was considered to indicate significance.

2.7. Molecular enrichment analysis

In order to find the appropriate miRNA for CLL, the miRWalk database was used. Moreover, to perform molecular enrichment analysis on *miR-193b-3p* and *miR-376a-3p*, several *in silico* online databases were used. Briefly, miRecords and miRTarBase databases were employed to find predicted and validated targets of *miR-193b-3p* and *miR-376a-3p* [21, 22].

3. Results

3.1. Clinical and biological features of patients

In the current study, 30 patients with CLL (mean age: 60.87 ± 1.591 years, range: 45-81 years, 18 males and 12 females, mean of disease duration: 1 ± 0.67 years) and 30 healthy individuals (mean age: 44.03 ± 1.952 years, range: 25-68 years, 21 males and 9 females) were studied. Patients and controls were sufficiently matched in terms of age and sex (P = 0.11 and 0.99, respectively). It was observed that, in the patients, the levels of some variables associated with CLL, including WBC, RBC, monocyte, lymphocyte, and platelet counts, were higher than the normal values. However, there was no significant difference between the RBC levels in the healthy individuals and patients (Table 1).

Table 1: Hematological parameters of patients with CLL.

Characteristics	Control	CLL	P-value
Number of subjects	30	30	-
Mean number of WBCs	7.077 ± 0.3239	63.87 ± 16.94	0.001
Range (cells per mcL/10^3)	4.5-9.9	6.03-451	
Mean number of RBCs	4.564 ± 0.1539	4.531 ± 0.1661	0.8835
Range (cells per mcL/10^6)	3.29-5.98	1.6-5.7	
Mean number of Monocytes	0.4463 ± 0.04840	5.958 ± 2.011	0.0081
Range (cells per mcL/10^3)	0.04-0.7	0.43-45.19	
Mean number of Lymph	2.530 ± 0.1377	43.67 ± 15.34	0.009
Range (cells per mcL/10^3)	1.2-3.7	2.38-408.03	
Mean number of PLTs	302.3 ± 17.85	135.9 ± 8.493	< 0.0001
Range (mcL/10^3)	155-420	21-273	

WBCs: white blood cells, RBCs: red blood cells, Mono: monocytes, Lymph: lymphocytes, PLTs: platelets. $p \leq 0.05$ was considered significant. Data were expressed as mean ±SD.

3.2. *miR-193b-3p* expression

The expression of *miR-193b-3p* was evaluated by quantitative real-time PCR in patients with CLL (n = 30) and healthy individuals (n = 30). The Ct values of real-time PCR were used for quantification of relative gene expression by the $2^{-\Delta\Delta Ct}$ method. A significant reduction was observed in the expression of *miR-193b-3p* in patients with CLL compared to that in healthy individuals with a $P < 0.0001$ (Figure 1). Thus, our results suggest that *miR-193b-3p* may be useful as a novel biomarker for patients with CLL.

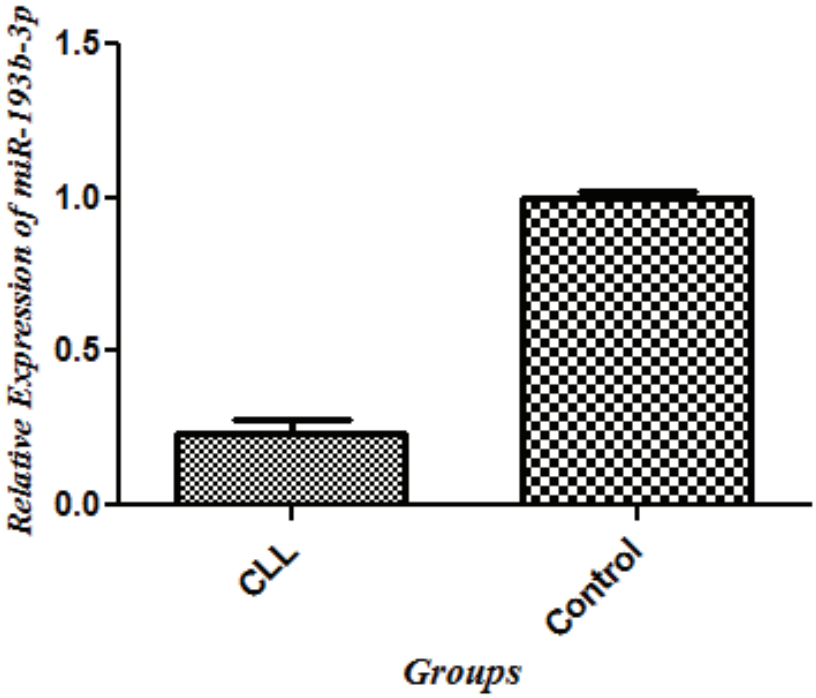

Figure 1: Relative expression of *miR-193b-3p* in patients with CLL and healthy individuals. Relative quantification for the *miR-193b-3p* was significantly different between CLL and control groups with a $P < 0.0001$. Error bars indicate SE.

3.3. *miR-376a-3p* expression

To explore the potential roles of *miR-376a-3p* in patients with CLL, we examined the expression level of *miR-376a-3p* in patients with CLL versus healthy individuals by qRT-PCR. Our results indicated that the expression level of *miR-376a-3p* was significantly downregulated in the patients with CLL compared to that in healthy individuals ($P< 0.0001$) (Figure 2). These results show that *miR-376a-3p* was significantly decreased in the cancer patients and probably plays an important role in CLL.

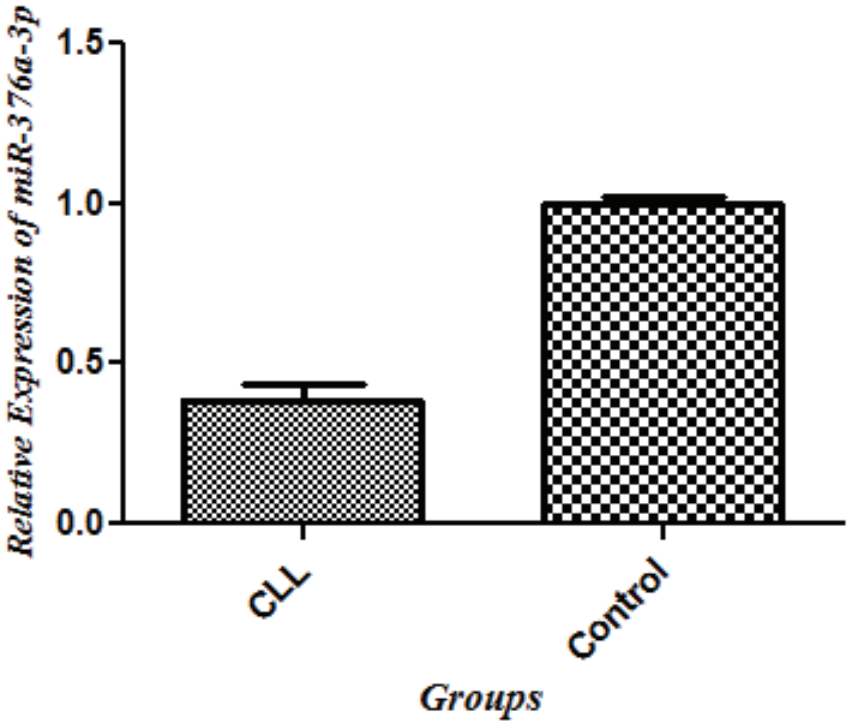

Figure 2: Relative expression of *miR-376a-3p* in patients with CLL and healthy individuals. Relative quantification for the *miR-376a-3p* was significantly different between CLL and control groups with a $P < 0.0001$. Error bars indicate SE.

3.4. Molecular enrichment analysis

Using the miRecords database, 76 and 34 mRNAs were identified as predicted targets of *miR-193b-3p* and *miR-376a-3p*, respectively (Supplementary Table 1 & 2). All predicted targets were confirmed by at least five prediction databases. Similarly, using the miR-TarBase database, 9 and 5 mRNAs were obtained as validated targets of *miR-193b-3p* and *miR-376a-3p*, respectively. The validated targets of *miR-193b-3p* that were obtained from the miRTarBase database included *CCND1, PLAU, MCL1, ETS1, MAX, KRAS, RAD51*, *NF1*, and *SMAD3*. Five validated targets were also identified for *miR-376a-3p*, including *PIK3R1*, *IGF1R, Pcna KLF15*, and *CASP8*. All the validated targets in the miRTarBase database were supported by strong experimental evidences, including reporter assays, western blots, and quantitative real-time PCR.

4. Discussion

Previous studies have indicated that prognostic prediction in CLL is based on V-gene mutations (CD38 and ZAP-70) and various types of chromosomal deletions, such as 17q13, 6q21, 11q23, and 13q14 in CLL [23]. Despite the progress made in monitoring

such patients, an accessible and reliable biomarker for diagnosis is still lacking. Early diagnosis of CLL would enable us to design more effective therapies. Circulating miRNAs could represent the available biomarkers for CLL [24]. miRNAs are well-known factors that affect the clinical phase of CLL. CLL was among the first human cancers discovered that was clearly related influenced by changes in miRNA expression. Deletion of *miR-15a* and *miR-16* in CLL was found to result in the overexpression of the *BCL-2* gene [25]. In order to develop an miRNA-based biomarker for CLL or any other disease, one of the most important prerequisites is the ability to quantify miRNAs from a variety of samples with sufficient sensitivity and reproducibility. However, the function and molecular mechanism of *miR-193b-3p* and *miR-376a-3p* in CLL disease are yet unknown. In this study, *miR-193b-3p* and *miR-376a-3p* were selected from miRWalk database, as these two miRNAs are involved in CLL disease. Subsequently, the expression of *miR-193b-3p* and *miR-376a-3p* was assessed by quantitative real-time PCR in 30 patients with CLL and 30 healthy individuals. The data revealed a reduced expression of both the miRNAs in patients with CLL compared to healthy individuals. According to our results, down-regulation of *miR-193b-3p* and *miR-376a-3p* may have functional importance and be associated with poor prognosis of CLL. Changes in the expression of *miR-193b-3p* have been associated with various human cancers such as ALL, prostate cancer, and ovarian cancer [13–15]. Mets et al. suggested that the novel tumor-suppressor, *miR-193b-3p*, can cooperate with NOTCH1 in the suppression T-ALL, by targeting the MYB oncogene in T-ALL [13]. Rauhala et al (2010) reported that *miR-193b* expression was decreased with increased methylation in clinical prostate cancer when compared to benign prostatic hyperplasia. This miRNA is an epigenetically regulated putative tumor suppressor in prostate cancer [14]. Furthermore, it has been shown that down-regulation of *miR-193b* in ovarian cancer cell lines can play a role as a novel biomarker for ovarian cancer [15]. On the one hand, the deregulation of *miR-193b* probably affects cell growth in colon cancer through the *TGF*-β and *SMAD3* signaling pathways [26]. On the other hand, evidence has demonstrated that the expression of *SMAD* proteins plays an important role in CLL development [27]. Thus, *miR-193b-3p* may be useful as a biomarker for early diagnosis of different cancers.

Herr et al (2017) reported that *miR-127* and *miR-376a* acted as strong tumor suppressors by in vivo targeting of COA1 and PDIA6 in a giant cell tumor of bone [16]. Meng et al (2015) demonstrated that *miR-7, miR-16, miR-25, miR-93, miR-182, miR-376a,* and *miR-429* show prognostic potential in serum of ovarian cancer patients [17]. Additionally, it has been suggested that down-regulation of *miR-376a* may contribute to the development of hepatocellular carcinoma by targeting p85α [18]. It has been proposed that *miR-376a*

regulates primordial follicle assembly by modulating the expression of proliferating cell nuclear antigen (*Pcna*) [28]. In CLL, *Pcna* levels are indicative of cell proliferation, clinical stage, and the lymphocyte doubling time [29]. Therefore, circulating *miR-376a-3p* can be used as a diagnostic and therapeutic biomarker. According to the molecular enrichment analysis, it can be assumed that *miR-193b-3p* and *miR-376a-3p* may have multiple target genes. Given that a miRNA has multiple targets, these different target genes may serve to raise important questions for future studies. Taken together, the findings of the current study showed the relevance of *miR-193b-3p* and *miR-376a-3p* to CLL. Consequently, in the future, *miR-193b-3p* and *miR-376a-3p* may be used as potential biomarkers for early diagnosis of patients with CLL. Understanding the complexity of miRNAs may open up a new vista to find biomarkers for clinical diagnosis of cancer and to monitor efficacy of various therapies. In conclusion, the present study suggests the role of *miR-193b-3p* and *miR-376a-3p* in CLL disease. Our data revealed that the expression levels of these miRNAs were downregulated in CLL. Thus, our results provide new insights regarding miRNAs, and *miR-193b-3p* and *miR-376a-3p* might serve as potential therapeutic targets or prevent disease progression. Although, it can be assumed from our results that *miR-193b-3p* and *miR-376a-3p* may have tumor-suppressing roles in CLL, more studies on larger scales are required to confirm this concept.

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Conflict of Interest

The authors declare that they have no conflicts of interest.

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