

Original Article

Expression Profiles of *miR-93* and *miR-330* in Iranian Patients with Chronic Lymphocytic LeukemiaBehnaz Nateghi¹ M.Sc., Elahe Shams² M.Sc., Parisa Behshood³ Ph.D., Sima Fathollahzadeh⁴ M.Sc., Mansoor Salehi^{5*} Ph.D.

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A B S T R A C T

Article history

Received 1 Sep 2018

Accepted 11 May 2019

Available online 31 May 2019

Key words

Chronic lymphocytic leukemia

microRNA

*miR-93**miR-330*

Background and Aims: Chronic lymphocytic leukemia (CLL) is the most common adult human leukemia. MicroRNAs (miRNAs) are small non-coding RNAs that regulate gene expression. Research has shown that in CLL, microRNAs can have function as oncogenes or tumor suppressors. Some studies demonstrated that the expression of microRNA-93 (*miR-93*) and microRNA-330 (*miR-330*) have been changed in several cancers, including lung, prostate, and colon cancer. We aimed to elucidate the changes in *miR-93* and *miR-330* expression in CLL patients in comparison with controls.

Materials and Methods: In this case-control study, the expression levels of *miR-93* and *miR-330* was evaluated in 30 CLL patients who had referred to Omid Hospital, Isfahan, Iran, and 30 controls in peripheral blood mononuclear cells using reverse transcription quantitative PCR (RT-qPCR).

Results: The expression of *miR-93* and *miR-330* were found to significantly increase in CLL patients compared with controls ($p < 0.0001$).

Conclusions: The findings indicated that *miR-93* and *miR-330* are probably the novel potential biomarker for early diagnosis of CLL, at least in Iranian patients. However, for a decisive result, further investigations are warranted.

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Introduction

Chronic lymphocytic leukemia (CLL) is a monoclonal disorder characterized by a progressive accumulation of functionally incompetent lymphocytes [1]. CLL is the most frequent type of leukemia recognized in western countries, accounting for about one-third of all cases of adult leukemias [2]. The incidence of this type of leukemia is 30 percent among all blood cancers and is slightly more common in men than in women [3]. The epidemiological evidence demonstrated that 5-10 percent of CLL cases are associated with a family history [4]. Advancing the understanding of CLL molecular genetics may have therapeutic stratification based on molecular prognosticators [5]. MicroRNAs (miRNAs), a new class of endogenous small noncoding RNAs with 18-22 nucleotides, have been associated with several types of cancer [6, 7]. miRNAs mediate post-transcriptional gene regulation, leading to the degradation or translation repression of target messenger RNAs (mRNAs) by recognition of 3' untranslated region of mRNAs [8]. In cancers, miRNAs can function as regulatory molecules or as oncogenes by down-regulating tumor suppressors [9]. The *miR-15a* and *miR-16-1* were discovered in 2002 within the 13q14.3 deleted region in CLL [10]. miRNAs have pivotal roles in cellular and molecular processes related to CLL. However, the prognostic value of miRNAs is still unknown [11]. Some studies have shown the change in expression of *miR-93* and *miR-330* in several cancers including lung, prostate, and colon cancer [12-17]. Moreover, a previous study has suggested *miR-93* and *miR-*

330 as miRNAs related to major molecular genetic alterations in acute myeloid leukemia [18]. However, the molecular mechanisms of *miR-93* and *miR-330* in CLL are still unknown. In the current study, the expression of *miR-93* and *miR-330* was investigated in peripheral blood mononuclear cells (PBMCs) of CLL patients and controls by real-time PCR method for the first time. The aim was to determine whether the deregulated expression of *miR-93* and *miR-330* can be used as a diagnostic biomarker in CLL patients.

Materials and Methods

Totally, 60 samples including 30 patients diagnosed with CLL in the Omid Hospital (Isfahan, Iran) and 30 controls (both male and female) were selected for the study. CLL was diagnosed based on blood cell count, cell morphology, and clinical symptoms. 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 the National Cancer Institute (NCI) guidelines [19]. Four ml peripheral blood was collected in EDTA-containing tubes and transported on ice to the laboratory. The study was approved by the Ethics Committee of the Medical Genetics Research Center of Genome. Written informed consent was received from all participating subjects prior to sample collection.

Complete blood count

Complete blood count (CBC) test was assessed using CA&XN-Series TM Automated Hemato-

logy Analyses (Kobe, Japan). The Sysmex XN series made use of fluorescence flow cytometry. Some variables associated with CLL were determined by this device.

Peripheral blood mononuclear cells isolation

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 a lower density 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 were frozen at -70°C until the next step.

miRNA extraction

miRNA from PBMCs was extracted using miRNA Hybrid-R (Geneall, Seoul, Korea) based on the manufacturer's instructions. The quality of miRNA was determined at a 260/280 nm wavelength ratio measured by a NanoDrop spectrometer (Thermo Scientific, Waltham, MA, USA).

Complementary DNA synthesis and real-time PCR

Complementary DNA (cDNA) synthesis was made using a standard kit (Pars Genome, Tehran, Iran) according to the manufacturer's leaflet. The real-time quantitative PCR reactions were carried out using a Rotor-Gene 6000 system (Corbett Life Science, Mortlake, Australia) in a

total volume of 10 µl. Briefly, 20 ng µl⁻¹ of cDNA product was added to a master mix, including 10 pmol µl⁻¹ of *miR-93*, *miR-330* and *U6* (as a housekeeping gene) primers (Pars Genome) and 5 ml of SYBR premix ExTaq II (TaKaRa, Kusatsu, Shiga Prefecture, Japan). The conditions of PCR were as follows: 95°C for 15 min, 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s.

Statistical analysis

Real-time PCR data analysis was performed using the $\Delta\Delta CT$ method, where CT is the cycle threshold [20]. Data were then analyzed using SPSS software version 20 (Chicago, IL, USA). For all the tests, $p \leq 0.05$ was considered as the level of significance.

Results

Clinical and biological features of the participants

In this study, two groups were sufficiently matched in terms of age and sex ($P=0.11$, 0.99 ; respectively). The demographic status of the participants were as follows: 30 patients with CLL (mean age: 60.87 ± 1.59 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 ± 1.95 years, range: 25-68 years, 21 males and 9 females) (Table 1). Moreover, it was observed that, the levels of some variables including white blood cells, lymphocyte, and platelet counts were associated with CLL and were significantly higher in the cases than in the controls.

Table 1. Clinical and demographic characteristics of participants

Characteristics	Control	CLL	P-value
Number of subjects	30	30	-
Sex:			
Number of males	21	18	0.99
Number of females	9	12	
Mean age	44.03±1.95	60.87±1.59	0.11
Range (years)	25-68	45-81	
Mean of disease duration (years)	-	1 ± 0.67	-
Range (years)	-	0.5-2.5	
Mean number of WBC	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 lymphocytes	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 PLT	302.3 ± 17.85	135.9 ± 8.493	< 0.0001
Range (mcL/10 ³)	155-420	21-273	

WBC= White blood cell; PLT= Platelet.

p≤0.05 was considered significant. Data were expressed as mean±SD.

Relationship between the expression of *miR-93* and *miR-330* and CLL

Data analysis indicated a significant increase in the expression of *miR-93* in CLL patients compared with the controls. The relative quantitation (RQ) for the *miR-93* was significantly different in CLL and controls with a p-value of <0.0001 (Fig. 1). Additionally, the results demonstrated significant growth in the expression of *miR-330* in CLL patients

compared with the controls (p<0.0015) (Fig. 2). We observed that the CLL patients were associated with higher levels of both miRNAs expression compared with the controls. Therefore, we hypothesized that this overexpression of *miR-93* and *miR-330* in CLL compared to the control could be studied as a potential therapeutic target to inhibit CLL progression in the future.

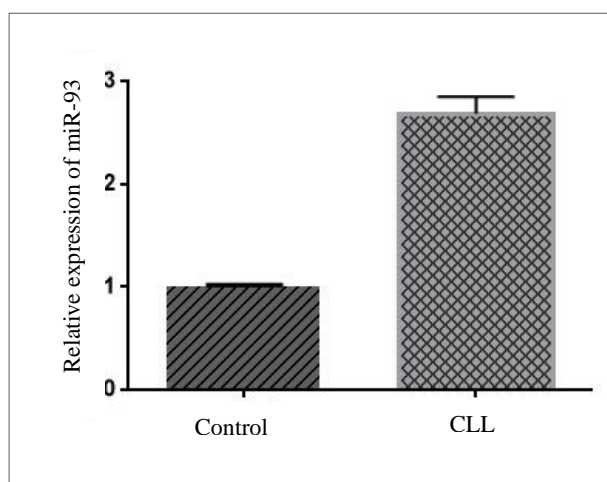


Fig. 1. Relative expression of *miR-93* in two groups: CLL (n=30) and controls (n=30). Relative quantitation (RQ) for the *miR-93* was significantly different in CLL and control (p<0.0001). Error bars show SE.

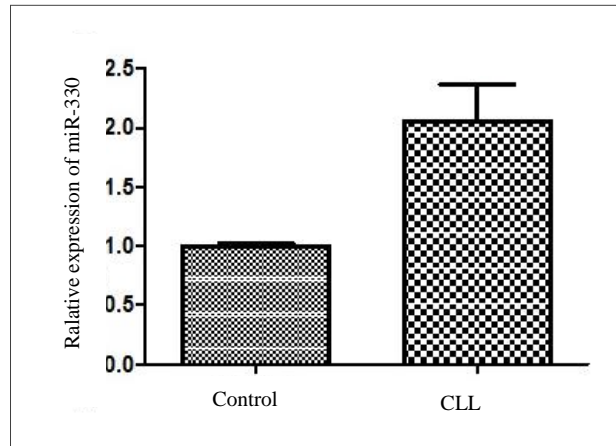


Fig. 2. Relative expression of *miR-330* in two groups: CLL (n=30) and controls (n=30). Relative quantitation (RQ) for the *miR-330* was significantly different in CLL and control ($p<0.001$). Error bars show SE.

Discussion

In the present study, our results indicated that the patients had a significantly higher expression of *miR-93* and *miR-330* compared with controls. According to previous investigations, prognostic prediction in CLL is based on V-gene mutations, CD38, ZAP-70, and deletion of the chromosome. The various types of chromosomal deletions, such as 17q13, 6q21, 11q23, 13q14 and 17p13, have been observed in CLL patients [21]. Appropriate non-aggressive biomarkers with high sensitivity and specificity are necessary for the diagnosis of the disease. miRNAs have emerged as biomarkers for the diagnosis and prognosis of different sorts of cancer. miRNAs have a deep impact on CLL development/progression [22-25]. Some studies have demonstrated the changes in expression of *miR-93* in several cancers, including lung cancer, prostate cancer, and colon cancer [12-14]. Also, the biological role of *miR-330* has been reported in some cancers including lung cancer, prostate cancer, and

colorectal cancer [15-17]. It has been suggested that *miR-93* and *miR-330* as a miRNAs are related to major molecular genetic alterations in acute myeloid leukemia [18]. However, as literature reveals, only one study has reported the involvement of *miR-93* in CLL. Expression of *miR-93* was significantly different in freshly isolated and cultured CLL cells carried out on a further group of patients using TaqMan real-time PCR [26]. In CLL, cancer cells originate from lymphoid cells, particularly B lymphocytes. These immature lymphocytes are observed in the blood. CLL is apperceived in lymph nodes, spleen, and other tissues [27]. The mechanisms for increased survival within tissues are not well understood but may provide novel targets for therapy [26]. Fabbri and Croce (2011) studied the role of microRNAs in lymphoid biology and disease. They suggested *miR-93* (high expression) and *miR-330* (high/low expression) as the most frequently de-regulated miRNAs in lymphoid malignancies

[28]. In the current study, the association of *miR-93* and *miR-330* expression with CLL disease was analyzed by RT-qPCR. Our results revealed that CLL patients have a significantly higher expression of *miR-93* and *miR-330* compared with controls. The current investigation is the first study on the association of *miR-93* and *miR-330* expression with CLL carried out using RT-qPCR (as a gold standard method for gene expression analysis). The identification of genetic factors involving in CLL can be useful for better understanding of the prognosis and treatment of CLL. However, the use of miRNAs as a biomarker still requires more investigations.

Conclusion

According to the results of this study, *miR-93* and *miR-330* deregulation is probably associated with CLL and poor prognosis can have functional importance. Also, these miRNAs can be a potentially valuable marker for early diagnosis of CLL. The identification of the genetic factors involving in CLL can be useful for better understanding of the pathophysiology and prognosis of CLL.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

This study was supported by Isfahan University of Medical Sciences. We thank all patients who funded to this investigation.

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