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Research Article

Circulating miR-95 Is a Potential Biomarker of Chronic Lymphocytic Leukemia

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Abstract

Background: MicroRNAs (miRNAs) have crucial roles in cellular and molecular processes related to different malignancies including chronic lymphocytic leukemia (CLL). Studies revealed altered miR-95 expression in several diseases. Long non-coding RNAs (lncRNAs) are a heterogeneous group of non-coding and regulatory RNAs.

The present study was conducted to investigate the association of miR-95 expression with CLL by quantitative real-time PCR.

Materials and Methods: Sixty samples, including 30 CLL and 30 healthy controls, were sampled during a period of 4 months. The expression of miR-95 was evaluated by quantitative real-time PCR in peripheral blood mononuclear cells from patients with CLL and in healthy subjects. Additionally, *in silico* pathway enrichment analysis was performed on validated and predicted targets of miR-95 in several databases, including miRecords and miRTarBase, while the interactions between predicted putative lncRNAs and genes and miRNA expression were examined with miRWalk.

Results: The expression of miR-95 was found to be significantly reduced in patients with CLL compared to that in healthy controls ($P < 0.005$).

Conclusion: miR-95 showed potential as a biomarker for the early diagnosis of patients with CLL. LncRNAs play a significant role in regulating cellular evolution, differentiation, and other processes and may be important regulators in tumorigenesis.

Keywords: chronic lymphocytic leukemia, long non-coding RNAs, miR-95, miRNA

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1. Introduction

Chronic lymphocytic leukemia (CLL) is one of the most common types of adult leukemia in the western world, defined by mature-appearing monoclonal B cells with CD5, CD19, and CD23 markers and decreased membrane-bound IgM and IgD levels [1]. Approximately 15,000 new cases of CLL are diagnosed in the USA each year [2]. B-lymphocytes mature in the bone marrow via rearrangement of the immunoglobulin

variable (V) gene, which is used as a B-cell antigen receptor leading to the formation of the code for an immunoglobulin molecule. This process involves rearrangement of the heavy chains of the VDJ gene encoding the receptor binding site [3]. The most common chromosomal abnormalities in CLL as detected by cytogenetics are a deletion at 13q (55%), 11q (18%), trisomy 12 (12–16%), and 17p (8%) [4]. MicroRNAs (miRNAs) are short non-coding RNA molecules 19–25 nucleotides long, contributing to the control of different biological processes. They regulate gene expression post-transcriptionally by binding to the 3′-untranslated region (UTR) or 5′-UTR of their mRNA targets [5, 6]. Changes in miRNA expression have been associated with many human diseases such as cancer and hematological malignancies and have been suggested as diagnostic biomarkers in patients with CLL patients [7, 8]. Changes in the expression of miR-95 have been associated with various human diseases such as colorectal cancer, lung cancer, osteosarcoma, and tumors by targeting sphingolipid phosphatase, brain metastasis of lung adenocarcinoma, and invasion-promoting apoptosis of glioma cells [9–15]. Studies have proposed that combined inhibition of PI3K/Akt and PTEN activity represents a new strategy for CLL treatment [15]. Some studies have supported a model in which miR-95 targeting of *SGPP1* leads to increased cellular *S1P* levels, followed by activation of the PI3K-Akt pathway [11]. Long non-coding RNAs (lncRNAs) are a heterogeneous group of non-coding and regulatory RNAs with lengths of approximately 300 base pairs to 100 kilobases. The transcription and processing of lncRNAs appear to be similar to these processes in mRNA, including RNA polymerase II transcription, 5′-capping and polyadenylation. Studies have shown that numerous lncRNAs play important roles in regulating cellular evolution, differentiation, and other processes. An important task of lncRNAs is to regulate the levels of miRNAs [16–18]. Some lncRNAs with binding sites for miRNAs and their presence in the cellular environment reduce the effects of miRNAs on targeted genes. In the presence of lncRNAs, inhibition of mRNAs by miRNAs is reduced, and these mRNAs will be translated into proteins [19, 20]. The role of miR-95 has not been determined in CLL previously. Therefore, We focused on miR-95, which has been predicted to have important roles in pathogenesis of CLL. Furthermore, pathway enrichment analysis was performed to predict the role of miR-95 in the pathogenesis of CLL, as well as relevant signaling pathways affected by this miRNA in CLL and the relationship of miR-95 with lncRNAs.

2. Materials and Methods

This study was performed using real-time PCR to examine the expression of miR-95 in peripheral blood mononuclear cells (PBMCs) from patients with CLL and in healthy subjects. The Ethics Committee of the Omid Hospital approved the protocol of this study.

2.1. Sample collection

Blood samples were collected from patients with early-stage disease and from random samples from healthy men and women. Sixty subjects including 30 patients with CLL,

diagnosed in the Omid Hospital (Isfahan, Iran), and 30 healthy controls were sampled according to the Internal Review and the Ethics Boards of the Isfahan University of Medical Sciences (Table 1). 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 guidelines [21]. Written informed consent was obtained from all participating subjects before sample collection. Six milliliters of blood were collected in EDTA-containing tubes from each subject. Immediately after sampling, the blood samples were transferred to the laboratory on ice.

TABLE 1: Clinical characteristics and hematological parameters of patients with CLL included in this study.

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 ± 1.952	60.87 ± 1.591	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 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 PLT	302.3 ± 17.85	135.9 ± 8.493	< 0.0001
Range (mcL/10^3)	155–420	21–273	

WBC: white blood cell, Lym: lymphocytes, PLT: platelet. $P \leq 0.05$ was considered significant. Data were expressed as the mean ± SD.

2.2. Blood cell count

Three variables associated with CLL, white blood cell counts (WBC), lymphocyte counts, and platelet counts, were assessed by CA&XN-Series TM Automated Hematology Analysis (Kobe, Japan). The Sysmex XN series uses fluorescence flow cytometry.

2.3. PBMC isolation

PBMCs were isolated by density gradient lymphoprep (Bio Sera, Kansas City, MO, USA) according to the manufacturer's protocol. Briefly, 4 mL of blood was diluted in the proportion of 1:1 with physiological saline and gradually added to 4 mL lymphoprep solution gradient. The prepared tubes containing the samples were centrifuged at 800 x*g* for 30 min at room temperature, and then the PBMCs were transferred from the middle phase into a 2-mL RNAase-free microtube. The samples were frozen at −70°C until RNA extraction.

2.4. RNA extraction

Total RNA from PBMCs was extracted using miRNA Hybrid-R (Geneall, Seoul, Korea) according to the manufacturer's instructions. miRNA quality was determined at a 260/280 nm wavelength ratio with a NanoDrop spectrometer (Thermo Scientific, Waltham, MA, USA).

2.5. CDNA synthesis and real-time PCR

cDNA was synthesized using a standard kit (Pars Genome, Tehran, Iran) according to the manufacturer's instructions. Real-time quantitative PCR was 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 was added to a master mix containing 10 pmol μL^{-1} of miR-95 and U6 (as a housekeeping gene) primers (Pars Genome) and 5 mL of SYBR premix ExTaq II (TaKaRa, Shiga, Japan). The PCR conditions were as follows: 95°C for 15 min, 40 cycles of 95°C for 15 s, 60°C for 30 s (annealing), and 72°C for 30 s (extension). PCR was followed by a melting curve program (60–75°C with a temperature transition rate of 1°C s^{-1} and continuous fluorescence reading). Negative controls lacking template were included with all reactions and all real-time PCR was performed in duplicate.

2.6. Statistical analysis

Real-time PCR data analysis was performed using the ΔΔCT method, where CT is the cycle threshold [22]. For statistical analysis, Graph Pad Prism statistical software, version 5.01 (Graph Pad, Inc., San Diego, CA, USA) was used. Normality was evaluated by the Kolmogorov–Smirnov test. The independent samples *t*-test was applied to analyze data between groups. For all tests, $p \leq 0.05$ was considered as the level of significance.

2.7. Systematic pathway enrichment analysis

In silico molecular signaling pathway enrichment analysis was conducted for known and predicted targets of miR-95 in several databases including miRecords and miRTarBase [23, 24]. Analysis was then conducted using the Database for Annotations, Visualization, and Integrated Discovery (DAVID), version 6.7 [25]. miRwalk software was used to predict interactions between lncRNAs and miR-95, which suggested putative lncRNAs interplay with gene and miRNA expression [26].

3. Results

3.1. Clinical and biological features of patients

In this study, 30 patients with CLL and 30 healthy controls were enrolled. The major clinical and biological features of the patients with CLL are shown in Table 1. The values for three variables associated with CLL, WBC, lymphocyte, and platelet counts, were higher than the reference interval in 60%, 93.3%, and 43.3% of patients, respectively.

3.2. miR-95 expression

The expression of miR-95 was evaluated by quantitative real-time PCR in the two groups (patients with CLL and healthy controls). The Ct values of real-time PCR were examined by the $2^{-\Delta\Delta Ct}$ method. The relative quantification was significantly different between the two groups (P-value < 0.005–2.7-fold) (Figure 1). Data analysis revealed a significant reduction in the expression of miR-95 in patients with CLL compared to that in healthy subjects.
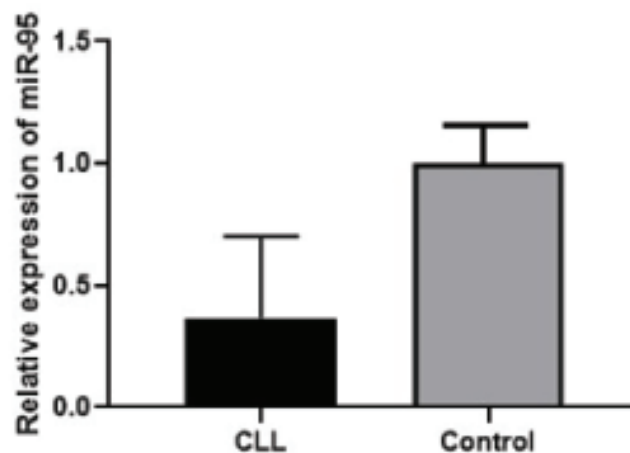

Figure 1: Relative expression of miR-95 in patients with CLL and healthy subjects. Relative quantification for the miR-95 was significantly different between CLL patients (n = 30) and control groups (n = 30) with a P < 0.005. Error bars show SE.

3.3. Molecular enrichment

Ten mRNAs were identified as targets of miR-95 using the miRrecords database. Predicted targets were confirmed by at least five prediction databases (Table 2). Validated targets enriched from the miRTarBase database were supported by experimental evidence including reporter assay, western blotting, and quantitative real-time PCR, which only revealed two genes: CELF2 and SNX. One of the important tasks of lncRNAs is to regulate miRNA levels. LncRNA binding site predictions were conducted using the miRWalk algorithm and predicted interactions of miR-95 and lncRNAs are shown in Supplementary Table 1. LncRNA interactions with miR-95 obtained from the 4 different algorithms are shown in Supplementary Table 2.

TABLE 2: Ten predicted targets in the miRecords database for miR-95[22].

Predicted targets	miRecord score
TTC3	5
YTHDF1	5
EID1	5
MGAT4C	5
CALM1	5
LAMC3	5
UBE4B	5
SNX1	5
IL1RAP	5
DPH4	5

4. Discussion

In this study, the expression of miR-95 was determined by quantitative real-time PCR in patients with CLL (n = 30) and healthy controls (n = 30). Our results revealed that patients with CLL express significantly lower levels of miR-95 compared to healthy controls. The importance of miRNA in CLL was demonstrated in 2002 when Calin et al found that miR-15a and miR-16-1 are encoded in chromosome 13q14, a region deleted in more than half of CLL cases, suggesting a link between these miRNAs and CLL [27]. However, the relationship between miR-95 and CLL has not been reported. Lisio et al. evaluated miRNA signatures in B-cell lymphomas and suggested that miR-95 expression is decreased in patients with CLL [28]. However, previous studies revealed a change in the expression of miR-95 as a tumor-suppressor in several cancers. It has been suggested that downregulation of miR-95-3p is associated with the poor prognosis of patients with osteosarcoma. MiR−95−3p is a therapeutic target and *CELF2* a potential tumor suppressor [14]. miR-95 may be a valuable diagnostic or prognostic marker for patients with osteosarcoma [12]. MiR-95 by its virtue of targeting *SNX1* was found to be a new oncogenic miRNA in colorectal cancer and was detected as an oncogene in non-small cell lung cancer cells by *SNX1* [9, 29]. Huang et al suggested a model in which miR-95 targeting of *SGPP1* leads to increased *S1P* levels, with subsequent activation of the PI3K-Akt pathway, resulting in cell survival and duplication [13]. Targeting inhibition of miR-21 and miR-95 may inhibit tumor growth by enhancing PTEN, SNX1, and SGPP1 expression and inhibiting Akt phosphorylation [10]. Moreover, Qin et al proposed that down-regulation of oncogenic miR-95, Akt, and SGK1 and blockage of phosphorylation of the Akt are associated with CRC [9]. This may be attributed to the fact that circulating miR-95 can be used as a diagnostic and therapeutic biomarker. Thus, miR-95 may be useful as a biomarker for the early diagnosis of patients with CLL.

LncRNAs are a large class of transcripts that represent over half of the mammalian non-coding transcriptome. LncRNAs may act as important regulators of tumorigenesis, signal, scaffolding, and transcriptional blockage [17, 18]. Ronchetti et al identified a 24-lncRNA-signature specifically deregulated in CLL compared to that in normal B-cells. This is related to some adverse prognostic markers, such as unmutated IGHV status,

CD38 expression, 11q and 17p deletions, and NOTCH1 mutations [30]. Blume et al suggested that NEAT1 and lncRNA-p21 are novel elements of the p53-dependent DNA damage response machinery involving lncRNAs in CLL [18]. LncRNA profiling of early-stage CLL revealed transcriptional changes related to clinical outcomes [30]. These results indicate the relevance of lncRNAs in patients with CLL. However, additional studies are needed to support the precise role of lncRNAs in CLL.

5. Conclusion

In this study, we compared the transcript levels of miR-95 in patients with CLL and normal subjects. miR-95 was selected by pathway enrichment analysis. Our results showed that miR-95 expression was significantly downregulated in patients with CLL compared to that in healthy controls. These observations demonstrate that miR-95 can predict the response to treatment and is a potential marker for the early diagnosis of CLL and potential therapeutic target. This miRNA may be useful as a new biomarker for diagnosing CLL in Iranian patients.

Acknowledgements

Conflict of interest

The authors declare no conflict of interest.

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