

Serum Expression of Seven MicroRNAs in Chronic Lymphocytic Leukemia Patients

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Ehsan Farzadfar¹
Tahereh Kalantari²
Gholamhossein Tamaddon^{3,4}

¹School of Paramedical Sciences, Shiraz University of Medical Sciences, Shiraz, Iran;

²Department of Medical Biotechnology, School of Paramedical Sciences, Shiraz University of Medical Sciences, Shiraz, Iran;

³Department of Clinical Laboratory Sciences, School of Paramedical Sciences, Shiraz University of Medical Sciences, Shiraz, Iran; ⁴Diagnostic Laboratory Sciences and Technology Research Center, School of Paramedical Sciences, Shiraz University of Medical Sciences, Shiraz, Iran

Purpose: MicroRNAs are small single-strand noncoding RNAs that can be deregulated in a variety of cancers. Over the past few years, multiple markers have been discovered in chronic lymphocytic leukemia (CLL). Among these, miRNAs seem to have important roles in the pathogenesis of CLL. The development and validation of miRNA-expression patterns as biomarkers should have a significant impact in cancer diagnosis, therapeutic success, and increasing the life expectancy of patients. In this study, to specify the utility of circulatory miRNA expression as noninvasive and useful biomarkers for CLL, we analyzed the dysregulation of seven miRNAs: miR30d, miR25-3p, miR19a-3p, miR133b, miR451a, miR145, and miR144 in CLL-patient sera.

Methods: Thirty untreated patients with flow-cytometry confirmation of CLL were chosen. Serum samples were collected from 30 newly diagnosed CLL patients. Fifteen healthy samples were taken for comparison as controls. RNA was extracted using Trizol. RNA from CLL patient specimens was compared to controls with real-time PCR.

Results: Seven miRNAs were differently expressed between CLL and normal specimens using the comparative $2^{-\Delta\Delta Ct}$ method. miRNAs 133b, 25-3p, 451a, 145, 19a-3p, and 144 were overexpressed in sera obtained from CLL patients, and miRNA-30d was underexpressed in patient samples. Among these seven miRNAs, miR19a-3p and miR25-3p showed the most deregulation in CLL patients.

Conclusion: Real-time PCR is an applied means to perform high-throughput investigation of serum-RNA samples. We assessed the expression of seven miRNAs in CLL patients by this method. The results demonstrated that the use of miRNA-expression profiling may have an impressive role in the diagnosis of CLL. In addition, miRNA 19a-3p and 25-3p are known oncogenes with therapeutic and potential biomarkers.

Keywords: chronic lymphocytic leukemia, circulatory microRNAs, noncoding RNA, real-time PCR

Introduction

B-cell chronic lymphocytic leukemia (CLL) is the most common leukemia in adults, and is characterized by the expansion of CD5⁺/CD19⁺/CD23⁺ B lymphocytes.^{1,2} CLL has a heterogeneous clinical course in which patients show an accumulation of a malignant clone of B lymphocytes in their bone marrow, lymphatic tissue, or bloodstream.³ Several prognostic factors predicting the clinical course have been defined for CLL patients. There are some poor prognostic factors, such as mutations in *IVGH*, high-level expression of ZAP70 and surface protein CD38, and chromosomal aberration indicating a poor prognosis. On the other hand, patients with mutated *IGVH* or those lacking ZAP70 have a good prognosis.⁴⁻⁶

Correspondence: Gholamhossein Tamaddon
Building 99, Narvan Street, Shiraz
74616-86688,
Tel +98 71 3227 2315
Fax +98 71 3227-0238
Email tamaddon.g@gmail.com

Clinical signs at the time of diagnosis are widely variable, and present as an indolent or aggressive state with different output. A large percentage of CLL patients are asymptomatic, and there is no need for treatment. Tiredness, weakness, anemia, lymphadenopathies, and splenomegaly are the main clinical signs in symptomatic patients who should receive treatment. Blood-cell counts, morphology of peripheral blood smears, and immunophenotyping are used for early diagnosis of CLL patients, but for further diagnosis more evaluation is required, such as expression of ZAP70 and CD38, which correlate with *IGVH*-mutation status.^{7,8} Also, cytogenetic and fluorescent in situ hybridization (FISH) are performed for CLL subtypes.⁹ However, the association between expression of ZAP70 or CD38 with unmutated *IGVH* genes is not absolute.^{10,11} On the other hand, FISH and bone-marrow biopsy, the key tests to choose appropriate treatment for patients with CLL, are invasive and expensive procedures. Therefore, it seems that the search for new biomarkers for CLL is necessary.¹²

Apoptosis is a physiological cell-suicide program that is one of the hallmarks of CLL, and impaired apoptosis represents an important mechanism in clinical resistance to therapies.¹³ Therefore, the development of therapeutic strategies based on targeting apoptosis in CLL is a very important issue. One of the important biomolecules that regulate the apoptosis pathway are miRNAs. MicroRNAs are a class of conserved small (~22 nt) noncoding RNAs that regulate gene expression at a posttranscriptional level by blocking translation or degrading target mRNAs. They can regulate many target genes simultaneously.¹⁴ Human miRNAs are located in cancer-associated regions of the genome. It seems that miRNAs play an important role in the pathogenesis of various human cancers.^{15–17} Evidence showed miRNAs can act as a diagnosis and therapeutic biomarker.¹⁸ Recently CD49d expression reported as a marker to decide for first treatment still, yet miRNAs be able to tools for biological treatment in addition biomarker.^{19, 20}

The aim of this study was to investigate the expression of a group of miRNAs in CLL patients. Seven miRNAs — miR30d, miR25-3p, miR19a-3p, miR133b, miR451a, miR145, and miR144 — that target important genes in the apoptosis pathway and are deregulated in several hematologic disorders were chosen for this study to consider their possible role as biomarkers. In the current study, we report the expression levels of these miRNAs detected by RT-PCR in serum samples of CLL patients with existing clinical data.

Methods

Samples and Patients

In accordance with the approval and moral-satisfaction form from Shiraz University of Medical Sciences (IR.SUMS. REC.1395.S609), 30 newly diagnosed CLL patients with $>5,000/\mu\text{L}$ B lymphocytes in peripheral blood previously confirmed by flow cytometry between 2016 and 2017 were enrolled in this study. Inclusion criteria were clinical signs and diagnosis of CLL and first diagnosis with no previous treatment. Serum samples from 15 healthy volunteers were used as controls. The control group underwent medical examinations and did not show any hematologic or other cancerous diseases.

RNA Extraction and cDNA Synthesis

Total RNA was extracted from the serum samples using Trizol reagent according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). Concentrations of extracted RNA were determined using NanoDrop (Hellma, Denmark), and aliquots of the samples were stored at -80°C . Specific cDNAs of selected miRNA were synthesized using specific primers (Parsgenome, Tehran, Iran) and a cDNA-synthesis kit (Exiqon, Vedbaek, Denmark). First, polyA tail was added to miRNAs with polyA polymerase at 37°C . Following this, RT enzyme, reaction buffer, and miR-specific primers for cDNA synthesis were mixed with RNA polyA tail, incubated at 45°C for 60 minutes, and inactivated at 85°C for 1 minute.

Real-Time PCR

Aliquots of the cDNA were used for quantitative PCR with real-time PCR Master Mix (Exiqon using an ABI (Applied Biosystems) apparatus according to the manufacturer's instructions. Primer pairs were obtained commercially from Parsgenome). 5s rRNA was used as internal control and PCR normalization. qRT-PCR was run under conditions of initial denaturation at 95°C for 5 minutes, followed by 40 cycles of 95°C for 5 seconds, 63°C for 20 seconds, and 72°C for 30 seconds. All qRT-PCR tests were performed in duplicate.

Statistical Analysis

miRNA expression was calculated using the equation $2^{-\Delta\Delta\text{Ct}}$. Statistical analysis was performed by GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). miRNA expressions were compared between patient and control groups with *t*-tests. $P < 0.05$ was considered statistically significant.

Bioinformatic Analysis

Identification of putative and validated target genes among differentially expressed genes for all seven miRNAs studied was performed using web-based software. The corresponding gene, miRBase ID, and sequence of each miRNA in this study was assigned before analysis. The web-based software used to investigate the miRNA targets was miRTarBase (<http://mirtarbase.mbc.nctu.edu.tw>), miRecords (<http://c1.accurascience.com/miRecords>), TargetScan (<http://www.targetscan.org>), miRanda (<http://www.miRNA.org>), DIANA microT (<http://diana.imis.athena-innovation.gr/DianaTools>), and miRWalk (<http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2>).

Results

Analysis of Serum-miRNA Expression in CLL Patients and Healthy Controls

To considering circulating miRNAs as potential diagnostic markers, we evaluated correlations between the expression levels of seven circulating miRNAs (miR30d, miR25-3p, miR19a-3p, miR133b, miR451a, miR145, and miR144a) and of CLL diagnosis by comparison of CLL patients and healthy controls (Figure 1). To evaluate expression patterns of these seven miRNAs, we performed real-time PCR using the sera of 30 CLL patients and 15 healthy controls. Real-time PCR showed overexpression in miRNAs 25-3p, 19a-3p, 145,

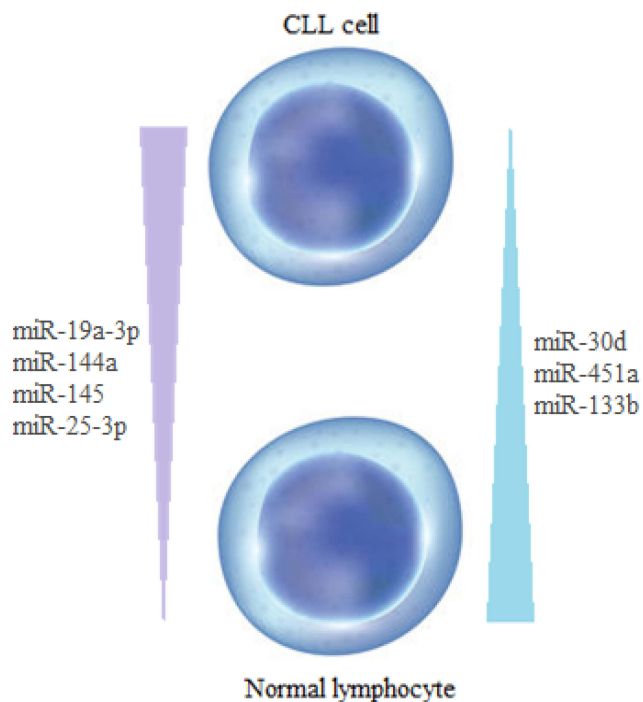


Figure 1 Differentially expressed miRNAs in CLL cells and normal cells.

and 144 ($P=0.0001$, $P=0.0007$, $P=0.023$, $P=0.0244$) and lower expression of miRNAs-133b, 30d, and 451a ($P=0.044$, $P=0.038$, $P=0.0323$) in the sera of CLL patients compared to controls were found. Fold changes in median expression of the seven miRNAs in patient samples versus controls are given in Figure 2.

Circulating miR25-3p Significantly Overexpressed in CLL Patients

Comparison of expression levels of the miR25-3p in sera of CLL patients and the control group is shown in Figure 3. As expected, real time PCR results showed significant alteration in miR25-3p expression in CLL samples. This result indicated that miR25-3p expression was much higher in CLL patients than healthy individuals — 2.35-fold ($P=0.0001$).

Circulating miR19a-3p Expression Higher in CLL Patients Than Healthy Controls

miR19a-3p (a member of the 17–92 miRNA cluster) expression levels, were demonstrated to be overexpressed in CLL patients compared with the control group. miR19a-3p expression was significantly upregulated in the sera of CLL patients compared with healthy individuals — 1.965-fold ($P=0.0007$, Figure 3).

Discussion

Discovering miRNAs and being more aware of their functions has accelerated our understanding about the mechanisms of regulation of gene expression. By binding to the

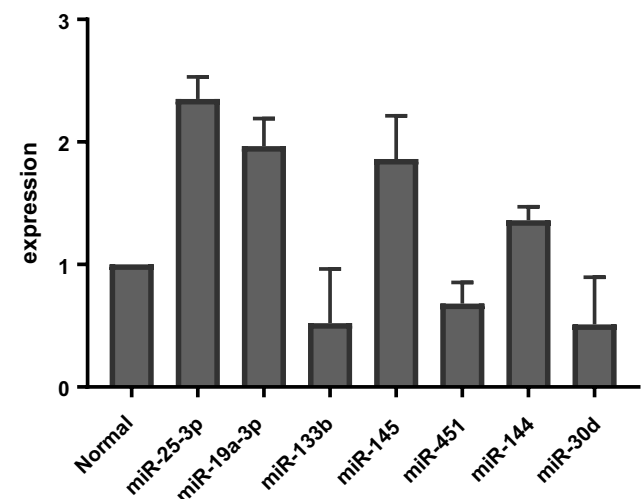


Figure 2 Median fold-change differences in alteration of miRNA expression between patient and control (normal). microRNAmiRNA expression analyzed using $2^{-\Delta\Delta Ct}$.

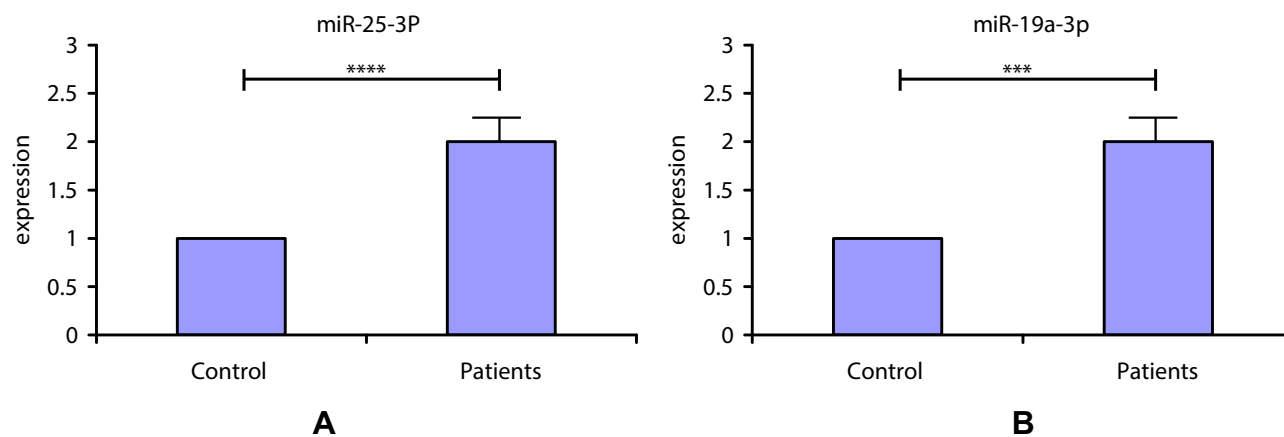


Figure 3 Deregulation of miR25-3p (A) and miR19a-3p (B) in CLL samples. Relative expression of miR-miR25-3p and miR-miR19a-3p in CLL samples compared to control. Expression normalized to 5s rRNA (endogenous control). Significant differences miR-miR25-3p between control and patients (**** $P=0.0001$) and miR-miR19a-3p (*** $P=0.001$). Results presented as mean \pm SD of three independent experiments, each performed in triplicate.

3' UTR of their targets, miRNAs can posttranscriptionally regulate gene expression.²¹ Some evidence has shown that miRNAs play important roles in a variety of vital cell processes, such as growth, development, proliferation, and death,^{22,23} as well as pathological processes, including developmental abnormalities, cardiovascular and autoimmune diseases, and cancers.^{24,25}

In this study, we showed different expressions of the seven miRNAs between CLL and normal serum using real-time PCR (Figure 1). Therefore, it seems that miRNA expression in serum samples can be turned into a noninvasive state to help diagnosis and treatment of CLL. We performed a pilot study to demonstrate that these miRNAs have a potential application as biomarkers for further studies in noninvasive CLL diagnosis and prospective studies with therapeutic targets.

As previously shown by other reports, many miRNAs are aberrantly expressed in CLL patients.^{26–28} In this study, we created an miRNA-expression profile that was achieved by investigating a set of 30 patients and 15 healthy controls.

Cytogenetic disorders in CLL patients show that the deficiency in apoptotic processes usually happens in this leukemia. Bioinformatic analysis of our seven selected miRNAs revealed that these miRNAs have multiple powerful targets in the apoptosis pathway (Table 1). Among these miRNAs, two of four overexpressed miRNAs (miR19a-3p and miR25-3p) are potential oncomiRs whose functions as an oncomiR have been reported in a variety of cancers.^{29,30} In addition, they have great potential as diagnostic and therapeutic tools for CLL patients.

Overexpression of miR25-3p has been demonstrated in a variety of cancers, such as papillary thyroid carcinoma,³¹ osteosarcoma,³² hepatocellular carcinoma,³² and lung cancer.³³ Heterogeneity of CLL has an impact on miR25-3p deregulation, as the expression level of miR25-3p has a correlation with such prognostic factors as cytogenetic disorders (lower levels in trisomy 12), *RBI*-gene deletion, and the age of patients.³⁴ Our results showed the expression of this miRNA increased 2.35-fold ($P=0.0001$) in patient samples. This alteration shows the high-impact roles of

Table 1 Intracellular Targets and Functions of miRNAs

miRNA	Expression Level	Chromosomal location	Putative Targets	Function
miR25-3p	High	Ch 7q22.1	<i>BCL2L11, TP53, PTEN, MDM2, SMAD7, CDK4</i>	Apoptosis, cell division
miR133b	Low	Ch 18q11.2	<i>BCL2L2, Casp9, MCL1, FAIM</i>	Apoptosis, cell differentiation
miR451a	Low	Ch 17q11.2	<i>AKT1, BCL2, MYC</i>	Apoptosis, invasion
miR30d	Low	Ch 8q24.22	<i>TP53, Casp3, SMAd7, SNAI1, MAP4K4</i>	Apoptosis
miR19a-3p	High	Ch 13q31.3	<i>PTEN, CCND1, SMAd4, BCL2L11, TGFBR</i>	Apoptosis, cell division
miR145	High	Ch 5q32	<i>CDKN, CCND1, MYC, CDK</i>	Cell division, apoptosis
miR144	High	Ch 17q11.2	<i>PTEN, TGFBR1, NOTCH1, BCL2L2</i>	Apoptosis

Abbreviation: Ch, chromosome.

miR25-3p as an oncomiR in the pathogenesis of CLL. TargetScan, miRanda, and miRTarBase analysis showed that it can potentially modulate the expression of important genes involved in regulating apoptosis and cell death (Table 1). All in all, this miRNA can be a suitable target to become a diagnostic biomarker or therapeutic tool in future studies.

miR19a-3p is a part of the 17–92 cluster, known as the oncomiR cluster.^{35,36} Overexpression of this miRNA cluster has been reported in several malignancies, such as acute myeloid leukemia,³⁷ multiple myeloma,³⁸ gastric cancer, and lung cancer.^{39,40} Bioinformatic analysis revealed that miR19a-3p has important putative targets, including *PTEN*, *BIM*, and *TGFBR*, in invasion, apoptosis, and cell death. In this study, high expression of miR19a-3p in CLL samples (1.965-fold, $P=0.0007$) shows the oncomiR roles of miR19a-3p in CLL malignancy. The important targets in the apoptosis pathway indicate that this miRNA can play a significant role in pathogenesis and progression of CLL.

Both miR144 and -145 were overexpressed in CLL samples (1.36- and 1.86-fold, $P=0.024$, $P=0.023$), which is in accordance with their putative and validated targets, such as *PTEN*, *NOTCH1*, *CDKN1*, and *MYC*. We showed low expression of miR30d, 133b, and 451a (0.51-, 0.52-, and 0.68fold; $P=0.038$, $P=0.044$, $P=0.032$) was tumor-suppressive in the patient group, as previously demonstrated by other reports.^{41–43} Our data show that circulating miRNAs might have great potential as diagnostic and therapeutic biomarkers in CLL. Although our results suggest the mentioned roles for miR25-3p and miR19a-3p, further investigations are needed.

Conclusion

In the present study, we used real-time PCR to assess the expression of seven circulating miRNAs — miRNAs 30d, 25-3p, 19a-3p, 133b, 451a, 145, and 144 — in the sera of CLL patients. The results showed these miRNAs were dysregulated in CLL samples compared with healthy controls. We also showed that miR19a-3p and miR25-3p were significantly increased in CLL patients and that they can play important roles in the pathogenicity of CLL.

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Disclosure

The authors report no conflicts of interest in this work.

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