



# Circulating miRNAs can serve as potential diagnostic biomarkers in chronic myelogenous leukemia patients

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## ABSTRACT

**Introduction:** Chronic Myelogenous Leukemia (CML) is a myeloproliferative disorder described as a malignant blood disorder by accounts for 15–20% of all adult leukemia. MicroRNAs (miRNAs) play an important role in post-transcriptional regulation of gene expressions. Expression level of tumor suppressor-miRNAs, described as miRNAs that target the oncogens, can contribute to diagnosis and prognosis of some malignant disorders including CML. We theorized that according to the excessive proliferation and alteration in miRNA expressions, there could be a change in the expression of miRNAs in plasma carried by exosomes.

**Methods:** We consequently decided to detect the differences between normal and aberrant miRNA expression in human plasma sample to find out the possibility of diagnosis by these alterations. The expression of candidate miRNAs were compared using RNA extracted from the plasma of 50 patients, as well as 30 healthy individuals. We analysed the plasma miR-16-1, miR-20, miR-106, miR-126, miR-155, miR-222, and miR-451 expression levels in CML patients by individual real-time quantitative RT-PCR.

**Results:** All selected miRNAs were found to be upregulated in newly diagnosed CML patients compared to the control, while upregulation of only three (miR-20, 106 and 222) were significant (17.4, 19 and 74.95 fold change, respectively;  $p < 0.0001$ ).

**In conclusion:** microRNAs have a potential use in treatment of CML, as they can target the genes involved in cell cycle, MAPK, growth inhibition, TGF beta, and p53 signaling pathways. Therefore, these miRNA signatures provide the basis for their utilization as biomarkers in CML.

## 1. Introduction

Chronic Myelogenous Leukemia (CML), also known as Chronic Myeloid Leukemia, is a member of the Myeloproliferative disorders (MPD) family. It is described as a malignant blood disorder that accounts for 15–20% of all adult leukemia [1]. CML occurs as a result of neoplastic translocation of chromosomes 9 and 22  $t(9; 22)(q34; q11)$  in Hematopoietic Stem Cells (HSCs). This translocation ultimately leads to an abnormal chromosome known as Philadelphia (pH) chromosome [2]. Philadelphia chromosome then generates a fusion oncogene BCR-ABL1

tyrosine kinase protein is the primary cause of chronic phase of CML. However, additional molecular changes are required in chronic phases to progress to its accelerated phase [3]. Besides, due to its tyrosine kinase activity, BCR-ABL1 protein affects the expression of numerous survival and anti-apoptotic pathways such as mutations in Tp53, RB, P16, overexpression of EVI1 and MYC, and also increases the activation of MAPK and PI3K. These regulations lead to an uncontrolled proliferation of HSC population, accompanied with an inhibition in the apoptosis process [4]. Tyrosine kinase inhibitor [5] drugs have changed CML patients' life. Among all TKI drugs, Imatinib, Nilotinib and

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Dasatinib have been chosen as the first-line therapy for pH+ CML patients in recent years [6–8]. However, 20% of those suffering from the chronic phase of CML are resistance to therapy which can rise up to 80% of those who are in the accelerated phase [9, 10]. MicroRNAs (miRNAs) are sequences up to 18–24 nucleotides, non-coding, single stranded ribonucleic acid that play an important role in post-transcriptional regulation of gene expressions [11]. Tumor suppressor-miRNAs are described by miRNAs that target the oncogenes. There are also onco-miRNAs that generally function in favor of cancer progression. Expression level of such miRNAs can cooperate in the process of diagnosis and prognosis of some malignant disorders like CML. Exploring this alteration in miRNA expression can define the changes in some gene regulations and expressions as well.

Based on an investigation in Kegg, TargetScan and miRWalk there are miRNAs associated with CML progression. From all, the miRNAs bellows have been selected due to their most targets related to CML progression. For instance, hsa-miR-16-1 is a tumor suppressor and modulates the MAPK signaling by targeting BCR, and P53 signaling by targeting P53 [12]. Moreover, hsa-miR-20 is a tumor suppressor which regulates MAPK signaling by targeting E2F3, BCR, KRAS, and P53 by CCND1 and P53, TGF beta by targeting TGFBR2 [13]. hsa-miR-106 is an onco-miR that regulates TGF beta signaling by targeting TGFBR2 also targets RUNX1 (AML1) which affects abnormality in growth inhibition [14]. hsa-miR-126 is a tumor suppressor [15] and regulates the MAPK signaling by targeting the MAPK8 and KRAS, TGF beta by targeting TGFBR2, P53 pathway by targeting P53 and CCND1, and abnormality in growth inhibition by targeting RUNX1. hsa-miR-155 is an onco-miR which regulates MAPK signaling by targeting E2F2, E2F3, BCR, CBL, KRAS, and SOS1, TGF beta by targeting TGFBR2 and SMAD4, P53 pathway by targeting CCND1 and P53, ErbB signaling by targeting PIK3R1, and abnormality in growth inhibition by targeting RUNX1 [16]. The hsa-miR-222 acts as an onco-miR when affects the PI3K-AKT signaling by targeting P27 and also works as a tumor suppressor by targeting the c-Kit in erythrocytes [17]. Overexpressed miR-222 can contribute the cell cycle progression of cancer cells by downregulating p27 in several types of human disease [18]. Hsa-miR-451 is a tumor suppressor [18, 19] which correlates inversely with the BCR-ABL [12]. We hypothesized that according to the excessive proliferation and alteration in miRNA expressions, there could be a change in the expression of miRNAs in plasma carried by exosomes or other exosome-independent mechanisms such as bounding to Ago2 protein [20]. Peng et al., also showed that patients with non-small cell lung cancer represent unique plasma exosomal miRNA profiles. They showed Hsa-miR-320d, hsa-miR-320c, and hsa-miR-320b to have a potential role in predicting the efficacy of immunotherapy in advanced non-small cell lung cancer [21]. Therefore, we carried out a study to detect the differences between normal and aberrant miRNA expression in human plasma sample to find out the possibility of diagnosis by these alterations.

## 2. Material and methods

### 2.1. Database of miRNA prediction and selection

All the miRNAs were selected using databases Target Scan version 5.1 ([www.targetscan.org](http://www.targetscan.org)), PicTar ([pictar.mdc-berlin.de](http://pictar.mdc-berlin.de)) and miRWalk. Besides, all the cell signaling pathways targeted by the selected miRNAs were analyzed using KEGG ([www.genome.jp/kegg/](http://www.genome.jp/kegg/)) and DAVID (Database for Annotation, Visualization and Integrated Discovery ([www.david.abcc.ncifcrf.gov](http://www.david.abcc.ncifcrf.gov))) databases. From all miRNAs shown to target the genes related to CML development, only miRNAs found to have the most targets were successfully chosen.

### 2.2. Primer design and stem loop

The sequences of 6 miRNAs were extracted from miRbase database

([www.mirbase.org](http://www.mirbase.org)). The selected human miRNAs included as follows; hsa-miR-16-1, hsa-miR-20, hsa-miR-106, hsa-miR-126, hsa-miR-155, hsa-miR-222. Considering the short length of miRNAs and the appropriate length of a typical primer, designing the primers for cDNA synthesis is almost an impossible task. However, adding a constant sequence at the end of the miRNA sequences makes primer designing for the reverse transcription (RT) attainable. In fact, by the use of universal stem loop [19] sequence and the selected miRNA sequences, RT primer design for cDNA synthesis was performed. Primers for real-time PCR were designed using the National Center for Biotechnology Information (NCBI) and Allele ID version 0.6. The reverse primer for Real Time-PCR application was designed based on the sequence of universal stem loop. The forward primer was designed based on each specific miRNA sequence (Table 1). Afterward, we aligned the primer sequences using the Basic Local Alignment Search tool (BLAST) to reveal the specificity of each specific primer.

### 2.3. RNA isolation, cDNA synthesis and quantitative real time-pcr for miRNAs

A complete blood count (CBC) was performed for 50 patients and 30 controls. Blood samples were kept on wet ice. Whole plasma separation process did not take more than an hour. Then, blood samples were centrifuged at 2000 rpm for 20 min in 4 °C. Next, total RNA were isolated from each plasma specimen using QIAzol (Qiagen), according to the manufacturer's protocol. cDNA synthesis was performed after designing RT primer for each predicted miRNA using the universal stem loop and miRNA specific sequences. Quantitative Real Time-PCR was carried out adopting 35 cycles at 95 °C for 15 s and 60 °C for 90 s. All miRNA reactions were normalized to the U6, which is the housekeeping gene for miRNAs in plasma specimen.

### 2.4. Specimen collection and ethic

Total peripheral blood sample were collected from 50 early diagnosed CML patients by the collaboration of Payvand Laboratory ([www.payvandlab.com](http://www.payvandlab.com)). Written informed consent was obtained from all study precipitants before they entered the study. The comparison groups were unrelated to the case group individuals. The patient's selection was considered based on the PCR positive results for t(9; 22) accompanied by standard microscopic morphology criteria confirming the CML. All selected patients were in chronic phase without other hematological disorders or former therapy. All total blood specimens was collected in tubes containing K3EDTA anticoagulant which were then transferred to research lab.

### 2.5. Statistical analysis

The expression data at miRNA level are described as mean  $\pm$  standard deviation (SD). Because of normal distribution of the data, the Student's *t*-test was used to compare fold change in expression. The correlation between study variables were calculated by Pearson's rank correlation coefficients. *P*-value of 0.05 was considered statistically significant. Statistical analysis was performed using SPSS version 12.0 software (Chicago, IL).

## 3. Results

### 3.1. Quantification of individual plasma miRNAs in CML

The expression of candidate miRNAs were compared using RNA extracted from the plasma of 50 patients, as well as 30 healthy individuals. We analyzed the plasma hsa-miR-16-1, hsa-miR-20, hsa-miR-106, hsa-miR-126, hsa-miR-155, hsa-miR-222, and hsa-miR-451 expression levels in CML patients by individual real-time quantitative RT-PCR. In this regard, all of selected miRNAs were found to be up-



**Table 2**

The expression levels of selected miRNAs between plasma and purified leukocytes from blood of CML patients.

miRNA	Classification	Analysis in plasma	Analysis in WBC	Previous data	Ref.
has-miR-16-1	Tumor suppressor	Up-regulated (No significant)	Up-regulated	Down-regulated in CLL and targets anti-apoptotic factor BCL2.	[12]
has-miR-20a	Tumor suppressor	Up-regulated	Up-regulated	Overexpressed in aggressive B-cell lymphomas and targets CDKN2A(p21).	[22, 33, 34]
has-miR-106	Onco-miR	Up-regulated	Down-regulated	Up-regulated in many cancer types, targeting TGFβ signaling	[14]
has-miR-126	Tumor suppressor	Up-regulated (No significant)	Up-regulated	Overexpression of miR-126 in acute myeloid leukemia (AML) patients associated with poor survival, and inhibition of miR-126 eradicates the CD34 + 38 stem/progenitor cells in AML	[15, 23, 24]
has-miR-222	Onco-miR/ tumor suppressors, depending on tumor system	Up-regulated	Up-regulated	Down-regulated in OTSCC and erythrocytes and targets c-Kit; Overexpressed in Prostate cancer and targets Bim; Overexpressed in breast cancer stem cells and targets ER-α FOXO3	[25]
Has-miR-155 and acts as onco-miR	Onco-miR (16)	Up-regulated (No significant)	Down-regulated	Targets MAF (c-maf) and induces lymphoproliferation	
Has-miR-451	Tumor suppressor	Up-regulated (No significant)	Down-regulated	Down-regulated in CML patients. Inverse correlation between BCR-ABL1 and miR-451 levels	[26, 27]

**Table 3**

Correlations between clinical data and miRNA fold variations.

miRNAs	Sex	WBC	Neutrophil	Lymphocyte	Monocyte	Eosinophil	Basophil	Age
hsa-miR-16-1	-0.020	-0.206	-0.166	-0.141	-0.112	0.035	0.047	-0.078
hsa-miR-20a	-0.137	-0.046	-0.148	-0.086	-0.114	-0.003	0.429*	-0.082
hsa-miR-106	p00.168	-0.221	-0.231	-0.234	-0.092	-0.310	-0.199	0.030
hsa-miR-126	-0.421*	-0.221	-0.502*	0.440*	0.317	-0.050	0.006	-0.189
hsa-miR-222	-0.142	-0.225	-0.331	-0.183	0.217	0.013	0.110	0.128
hsa-miR-155	-0.222	-0.007	-0.145	0.321	0.258	0.204	0.015	-0.424*
hsa-miR-451	-0.015	-0.023	-0.132	0.420	0.432	0.124	0.170	0.046

procedures such as biopsy or surgery. A plasma miRNA-based biomarker would allow the comprehensive analysis of diseases in a less invasive manner [30]. Therefore, the present study was aimed to evaluate the expression pattern of a panel of miRNAs (selected according to their role in targeting the hub genes in CML) and the molecular pathogenesis of CML and subsequently be applied for prognostic analysis of CML and appropriate therapeutic strategies of the disease.

In a previous study, we performed relative quantification of selected miRNAs' expression in leukocytes from the buffy coat of blood samples [31]. In this study, we compared the selected miRNAs' expression in plasma of early diagnosed CML patients. In this regard, all selected miRNAs were found to be up-regulated in newly diagnosed CML patients compared to control group, although up regulation of only three miRNAs (miR-20, 106 and 222) were significant ( $p < 0.0001$ ).

Quantitative real-time-PCR revealed significant up-regulation of miR-16-1, miR-20a, miR-106, miR-126, miR-155, miR-222, and miR-451 to different extents in comparison with the control group (Fig. 1). These miRNAs act as onco-miRs or tumor suppressors and are increased in other cancer types (Table 2). Previous studies on the expression pattern of miR-16-1 in newly diagnosed CML-CP treated with IM have shown that down-regulation of miR-16 can play important roles in myeloid leukemogenesis by regulating cell cycle and apoptosis, as well as targeting multiple oncogenes, including, BCL2, MCL1, CCND1, and WNT3A [32]. Inversely, in the present study and our previous experiment for CML, we surprisingly observed up-regulation of miR-16-1 in this particular disease state (Fig. 1). Moreover, our data showed that miR-20a is up-regulated in the chronic phase of CML, similar to aggressive B-cell lymphomas by targeting CDKN2A [33, 34]. We also showed that miR-106 is overexpressed in plasma sample of CML patients. Although there are no studies in line with our result in CML available, Garzon et al. showed that miR-106 is upregulated in the acute phase of the disease AML, suggesting that it might be characteristic of blast phase [22].

Confirmed increase of miR-126, miR-155, and miR-222 was identified in our experiment. In this line, Polakova et al. 2011 using leukocytes from 24 peripheral blood patient samples some miRNAs such as miR-222, miR-126, and miR-155 to be upregulated in samples of Blast Crisis, though not at Diagnosis, Hematologic relapse, Treatment Failure or Major molecular response [25].

Moreover, we also showed that miR-451 is overexpressed in plasma sample of CML patients in the chronic phase. miR-451 had a decreased expression in patients with CML at the time of diagnosis and in patients with hematologic relapse [27, 29]. In Ferreira et al. study, the miR-451 expression level found in total leukocytes of PB at diagnosis was reported to be associated with the response to IM therapy. They compared newly diagnosed CML-CP to CML-CP treated with IM and observed that miR-451 was up-regulated in patients who are responsive to treatment with IM, while it was shown that the miR-451 level may inversely correlate with the BCR-ABL in some CML patients [35].

In summary, many studies have identified miRNA expression profile for different kinds of diseases, especially cancer. Our data demonstrated that circulating miR-16-1, miR-20a, miR-106, miR-126, miR-155, miR-222, and miR-451 are deregulated in plasma of chronic phase of newly diagnosed CML patients. Although the exact regulatory mechanism remains unclear, circulating plasma miRNAs are detected to be within microvesicles which may help protect the miRNAs from RNase dependent degradation and may explain the observed up-regulation [36]. MicroRNAs have a potential use in treatment of CML, because they can target the genes that are involved in cell cycle, MAPK, growth inhibition, transforming growth factor beta and p53 signaling pathways. Therefore, these miRNA signatures provide the basis for their utilization as biomarkers in CML.

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**Table 4**

The miRNA and predicted targets containing target sites were further studied according to the PCT values in signaling pathway.

miRNAs	Targets	Definition	P <sub>CT</sub>	Pathway in CML
hsa-miR-20a	E2F3	E2F transcription factor 3	0.63	MAPK signaling
hsa-miR-16-1	E2F3	E2F transcription factor 3	0.38	MAPK signaling
hsa-miR-222	E2F3	E2F transcription factor 2	0.32	MAPK signaling
hsa-miR-126	MAPK8	mitogen-activated protein kinase 8	0.57	MAPK signaling
hsa-miR-20a	BCR	Breakpoint cluster region protein	0.56	MAPK signaling
hsa-miR-16-1	BCR	Breakpoint cluster region protein	0.73	MAPK signaling
hsa-miR-126	CRK	Proto-oncogene C-crk	0.68	MAPK signaling
hsa-miR-20a	KRAS	GTPase	0.79	MAPK signaling
hsa-miR-126	KRAS	GTPase	0.72	MAPK signaling
hsa-miR-16-1	KRAS	GTPase	0.84	MAPK signaling
hsa-miR-222	CBL	E3 ubiquitin-protein ligase	0.33	MAPK signaling
hsa-miR-126	TGFBR2	Transforming growth factor –beta receptor type-2	0.73	Transforming growth factor β
hsa-miR-20a	TGFBR2	Transforming growth factor –beta receptor type-2	0.39	Transforming growth factor β
hsa-miR-106b	TGFBR2	Transforming growth factor –beta receptor type-2	0.55	Transforming growth factor β
hsa-miR-20a	CCND1	Cyclin D1	0.65	p53 pathway
hsa-miR-16-1	CCND1	Cyclin D1	0.54	p53 pathway
hsa-miR-106b	CCND1	Cyclin D1	0.99	p53 pathway
hsa-miR-126	CCND1	Cyclin D1	0.49	p53 pathway
hsa-miR-126	RUNX1	Runt-related transcription factor 1 (AML1)	0.37	Abnormality in growth inhibition
hsa-miR-20a	RUNX1	Runt-related transcription factor 1 (AML1)	0.45	Abnormality in growth inhibition
hsa-miR-16-1	P53	P53	0.56	p53 pathway
hsa-miR-126	P53	P53	0.43	p53 pathway
hsa-miR-20a	P53	P53	0.79	p53 pathway
hsa-miR-222	CDKN1B	Cyclin-dependent kinase inhibitor 1B (p27)	0.50	Cell cycle

agencies in the public, commercial, or not-for-profit sectors.

## 8. Author contributions

FK performed the research; A.J., A.S., and E.J. analyzed the data and wrote the paper; F.K., M.M., and A.J. contributed the clinical sample collection as well as designed some of the study and, P.F. designed the research study.

## Declaration of Competing Interest

No competing financial interests exist.

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