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Evaluation of miRNA-126 Expression and Some Immunological Parameters as Diagnostic Biomarker in Chronic Myeloid Leukemia Iraqi Patients

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Abstract: Chronic Myeloid Leukemia (CML) is a hematologic malignancy characterized by the uncontrolled proliferation of myeloid cells in the bone marrow. Recent advancements in cancer research have highlighted the crucial role of cancer stem cells (CSCs) in tumor initiation, progression, and treatment resistance. The aim of this research is to investigate the potential of miRNA-126, CD33, and TNF-alpha as biomarkers for identifying and characterizing CSCs in a cohort of Iraqi patients with CML.The research methodology involves the collection of bone marrow samples from a cohort of Iraqi CML patients. Subsequently, various molecular techniques, including polymerase chain reaction (PCR) and flow cytometry, are employed to analyze the expression levels of miRNA-126 and CD33, in these samples. The choice of these biomarkers is based on their known associations with cancer stem cells and their relevance to CML.The results of this study showed that there were significant increases in miRNA-126 and CD33 ($p<0.05$) in a sample of Iraqi patients with CML compared to healthy people. It was concluded the dramatic increases in the expression of each parameter as shared above.

Keywords: Chronic myeloid leukemia (CML), Cluster Differentiation (CD33) MicroRNA (miRNA).

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Introduction

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. Unhealthy white blood cells have overproduced themselves. 15-20% of all adult cases of leukaemia are Chronic Myelogenous Leukaemia (CML), a myeloproliferative illness characterised as a malignant blood disorder (1).

Distinguishing feature of CML is the Philadelphia chromosome, which is created when the long arms of the 9 and 22 chromosomes are balanced reciprocally translocated [t (9; 22) (q34; q11)]. The BCR-ABL1 fusion gene, which produces the BCR-ABL oncoprotein, is produced by the union of the Abelson murine leukemia (ABL) gene on chromosome 9 and the breakpoint cluster region (BCR) gene on chromosome 22 (2). Afterward, the Philadelphia chromosome produces a fusion oncogene (3). The main factor causing the chronic phase of CML is the BCR-ABL1 tyrosine kinase protein. To advance to its accelerated phase, however, additional molecular modifications are needed in the chronic phases (4). MicroRNAs (miRNAs) are a group of non-coding RNA molecules

with a length of 19 to 25 nucleotides that participate in the regulation of gene expression after transcription (5). They also play an important role in many physiological processes, such as proliferation, differentiation, apoptosis, and hematopoiesis (6). The small non-coding RNAs (19-22nt) develop post-transcriptional regulation by mRNA cleavage or translation repression (7), which depended on the complementarity degree of miRNA-mRNA (8). Although miR-126 has similar patterns of expression and function in CML as in normal hematopoiesis, as noted that human CML Lin−CD34+CD38− (HSCs) and Lin−CD34+CD38−CD90+ (LTHSCs) cells had significantly lower miR-126 levels than their normal counterparts (9). As an important member of the miRNA family, microRNA-126 (miR-126), located within the seventh intron of epidermal growth factor-like protein 7 gene , was participated in a wide range of biological functions(10).

The cluster of differentiation (CD) markers are essential for recognizing and differentiating cells. Surface CD markers can express differentially in response to environmental factors and intracellular genetic alterations and serve particular purposes depending on the kind of cell (11).In spite of this, little is known about how CD33 is expressed on leukemic stem cells in chronic leukemias(12). Strong surface expression of CD33 was also seen on CML LSC from patients with imatinib-resistant illness in addition to imatinib-responsive disease (13)(14).

Materials and methods

Specimen collection

In this research, 40 Iraqi patient with chronic myeloid leukemia with ages ranging from 18 to 62 years who visited Baghdad teaching hospital between the 2022 and the end of November 2022 were included. Comparison of the patients with 40 volunteers, whose ages ranged from 19 to 65 who appeared to be good health. The blood was taken from the patients and healthy group. All of the study participants had their blood drawn using disposable syringes. Each individual had three milliliters of venous blood drawn, with one milliliter going into a gel tube to be used for the ELISA technique to measure expression of miRNA-126 and the level of CD33. The gel tubes were divided into aliquots and stored at - 20° C after being centrifuged for 10 minutes at 3000 rpm to separate the serum.

RNA purification

RNA was extracted from serum samples according to the protocol of TransZol Up Reagent. The serum (0.4ul) was mixed with 0.5 mL of TRIzol™ Reagent in each tube, and the lysate was homogenized by pipetting upand down multiple times.For each tube, 200 µl of chloroform was added to the lysate before securing the tube top. All mixtures were incubated for 3 minutes before being centrifuged at 10,000 rpm for 10 minutes to separate the mixture into a lower organic phase, interphase, and a colorless upper aqueous phase. The aqueous phase containing the RNA was transferred to a new tube. The resulting mixture was transferred to a spin column, centrifuged at 12,000 rpm for 30 seconds and the flow through was discarded and this step was repeated again. A volume of 500 µl of clean buffer (CB9) was added in to the spin column, centrifuged at 12,000 rmp for 30 seconds at room temperature and the flow through was discarded and this step was repeated again. A volume of

500 µl of wash bufer (WB9) was added into the spin column, centrifuged at 12,000 rpm for 30 seconds at room temperature and the flow through was discarded and this step was repeated again. The spin column was centrifuged at 12,000 rpm for 2 minutes at room temperature in order to completely remove remaining ethanol.The spin column was placed into a clean 1.5 ml of RNase free tube, 50-200 µl of RNase-free water was added into the spin column matrix and incubated at room temperature for 1 minute. Finally, RNA was eluted by centrifugation at 12,000 rmp for 1 minute and stored at -80 ºC.

Primers design

Primers design Using the NCBI Gene Bank database and miRBASE, the sequences retrieved and used as a template for primer designs, stem-loop structure considered for efficient detection and quantification (Table 1).

Primer preparation and optimization

A primer working solution was prepared from the lyophilized primer after dissolving in nuclease free water according to the manufacture to make a stock solution with a concentration of 100 µm for each primers and stored at (-23°C). A working solution with a concentration of 10 µm was prepared by mixing 10 µl of primer stock solution with 90 µl of nuclease free water to reach a final volume 100pmoles/µl and stored at (-20°C) until use.

Complementary DNA (cDNA) Synthesis for miRNA

Total RNA was reversely transcribed to complementary DNA (cDNA) using Easy Script® gDNA removel and cDNA synthesis super Mix Kit procedure was carried out in a reaction volume of 20 µl according to the manufacturer's instructions as shown in (Table 2). The total RNA volume to be reversely transcribed was 20 µl. The efficiency of cDNA concentration was assessed through the efficiency of qPCR conducted later on all stepes that associated with perfect yield reflecting efficient reverse transcription.

Quantitative real time PCR

The expression levels of miR-126 gene was estimated by qRT-PCR. To confirm the expression of target gene, quantitative real time qRT-PCR SYBR Green assay was used. The miRNA levels of endogenous control housekeeping gene were amplified and used to normalize the miRNA levels.The PCR master mix preparation as shown in (Table 3) and Real Time PCR Program thermal cycling conditions for miR-126, in (Table 4).

The expressions of the target were normalized by setting appropriate thresholds to obtain accurate CT values that were provided from the qRT-PCR instrument according to the following formula:

ΔCT= CT (gene of interest)-CT (housekeeping reference gene).

ΔΔCT = ΔCT (patient) -ΔCT (control)

The fold change value was then calculated from the following equation:

Fold change=2^(-ΔΔCT) normalized expression ratio.

Up-regulation of the target gene was identified by the positive result of the fold change, while negative results indicated downergulation of the target gene.

So, the results were expressed as fold change compared to the control sample which was considered the normal value and assumed to equal (1). CT values≥ 38 were considered unreliable and neglected.

Table (1): Sequences of the primer used in this study.

Primer Name	Direction	5'-3'	TM	GC %
miR-126	StemLoop	GAAAGAAGGCGAGGAGCAGATCGAGGAAGAAGAC GGAAGAATGTGCGTCTCGCCTTCTTTCAACTCTCAG	-	-
	F	GTACGGGGCCGAGCACT	59.5	70.6
	R	CGAGGAAGAAGACGGAAGAAT	57.3	55.6
U6	F	GCTTCGGCAGCACATATACTAAAAT	57.5	40
	R	CGCTTCACGAATTTGCGTGTCAT	59.7	47.8

Table (2): Reaction components of cDNA synthesis.

Component	Volume
Total RNA/MRNA	7 μl
Anchored oligo (dT) primer (0.5 /)	1 μl
Random primer (0.1/)	1 μl
RNAas free water	10 μl
Easy Script® RT/RI Enzyme Mix	1 μl
Total	20 μl

Table (3): Thermal cycle steps conditions.

	Step1	Step2	Step3
Temperature	25°C	42°C	85°C
Time	15min	10min	5sec
	Random Primer (N9)	Anchored oligo (dT)	Inactivate reverse transcriptase enzyme

Housekeeping gene amplification

Housekeeping gene (U6) was used as an internal control to be used in calculating the CT value. A qPCR reaction of amplification of housekeeping gene was done with the thermal profile shown in (Table 4).

Table (4): Thermal profile of U6 gene expression.

Step	Temperature	Duration	Cycle
Enzyme activation	94°C	30 sec	Hold
Denature	94°C	5 sec	40 cycle
Anneal/extend	62°C	15 sec	
Dissociation	55°C-95°C		

Genes expression of miR-126 and miR-155 qRT-PCR

A qPCR reaction of amplification of miR-126 and miR-155 gene was done with the thermal profile shown in (Table 5).

Table (5): Thermal profile of miR-126 gene expression.

Step	Temperature	Duration	Cycle
Enzyme activation	94°C	30 sec	Hold
Denature	94°C	5 sec	40 cycle
Anneal/extend	59°C	15 sec	
Dissociation	55°C-95°C		

Measuring the level of CD33 in the serum by ELISA

This assay employs the quantitative sandwich enzyme immunoassay technique after dilution as show in (Table 6). Antibody specific for CD33 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any CD33 present is bound by the immobilized

antibody. After removing any unbound substances, a biotin-conjugated antibody specific for CD33 is added to the wells. After washing, avidin conjugated Horseradish Peroxidase (HRP) is added to the wells. Following a wash to remove any unbound avidin-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of CD33 bound in the initial step. The color development is stopped and the intensity of the color is measured.

Table (6): Standard dilution for CD33.

Standard Concentration	Standard No.							
	7	6	5	4	3	2	1	0
8000ng/l	8000ng/l	4000ng/l	2000ng/l	1000ng/l	500ng/l	250ng/l	125ng/l	0 ng/l

Calculation of results

Average zero standard was subtracted from all readings. The duplicate readings of the positive control dilution were also average and plotted against their concentrations. Then, the best smooth curve through these points was drown to construct a standard curve CD33 concentrations for unknown samples from the standard curve were plotted. Samples producing signals greater than that of the highest standard should be further diluted and reanalyzed and then multiplying the concentration was found by the appropriate dilution factor as shown (Figure 1).

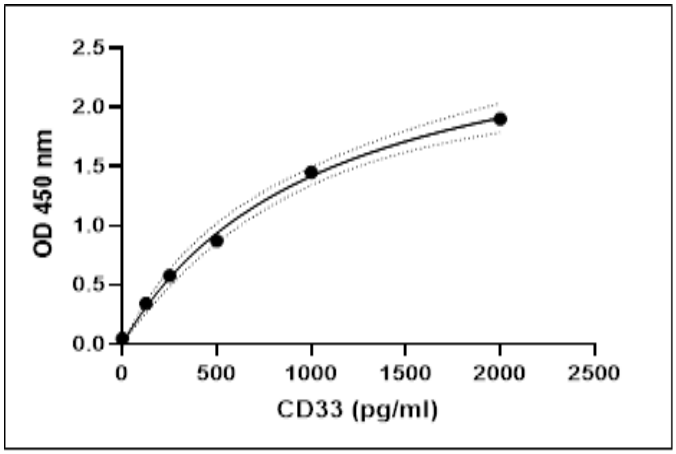

Figure (1): Standard curve of CD33.

Results and discussion

Quantitative genetic data analyses

The expression of miRNAs was compared using RNA extracted from the plasma of 40 patients, as well as 40 healthy individuals. analyzed miR-126 has high expression levels in CML patients by individual real-time quantitative RT-PCR. In this regard, selected miRNA-126 to be upregulated in newly diagnosed CML patients compared to the control group, were significant (17.7±9.48) change, $p<0.0001$, as show in (Table 7).

Table (7): The expression of mir -126 gene among patients and healthy controls.

ΔCT (Mean±SE)	P value	ΔΔCT (Mean±SE)	P value	Fold Change ($2^{-\Delta\Delta CT}$) Mean±SE
11.6±0.389	0.0012**	-1.74±0.389	0.0006**	17.7±9.48
13.4±0.340		0.118±0.340		2.55±0.561

Serum level of CD33

Leukemic cells have considerably greater levels of CD33 (444 ± 219) pg/ml than normal cells, which have a mean level (343 ± 78.8) pg/ml and a p-value of (0.0294), as shown in Table (8). This outcome is consistent with the discovery that immature stem cells exhibit high levels of CD33 expression and may act as a target for stem cells in CML, CD33 is a transmembrane glycoprotein belonging to the sialic acid-binding immunoglobulin-like lectin (Siglec) family (15).This study supports the findings that CD33 is a more myeloid marker and is frequently used to identify chronic myeloid leukaemia. However, CD33 expression may be aberrant in 10–20% of B- or T-lymphoblastic leukaemias or lymphomas (16).

(Table 8): Variables under research are compared between the patient and healthy control group.

Variables	Control (mean±SD)	Patients (mean±SD)	t – test (p – value)
CD33	343 ± 78.8	444 ± 219	0.0294*

Conclusion

The results of this study suggested that:

- Overexpression in miRNA-126 in patients compared to control suggest to serve as useful biomarkers for CML diagnosis and these microRNAs could serve as prognostic biomarkers, helping to identify patients at higher risk and tailor treatment strategies accordingly.
- CD33 can be used as biomarkers for diagnostic CML has advanced as understanding of the biology of the condition, identified prospective therapeutic targets, and improved patient management.

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