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The role of gene expression hypoxia-inducible factor-1a and serum level monocyte chemoattractant protein-1 in the incidence of chronic myeloid leukemia in Iraqi patients

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Abstract:

BACKGROUND: Chronic myeloid leukemia (CML) is a hematopoietic stem cell malignancy described by a translocation between chromosomes 9 and 22. There are many factors genetic or nongenetic effect on disease progression such as growth factors and transcription factors act as oncogenes or tumor suppressor genes.

OBJECTIVES: The purpose of this research was to investigate the role of hypoxia-inducible factor (*HIF1A)* gene expression with CML, as well as the role of monocyte chemoattractant protein-1 (MCP-1) as a predictive biomarker on disease progression.

MATERIALS AND METHODS: The current study consists of three groups: first group includes 50 newly diagnosed CML patients (females 22 and males 28), second group consists of 50 CML patients treated with tyrosine kinase inhibitor (TKI) with a complete molecular response (p210 *BCR-ABL* transcript levels ≤0.1% IS) (female 25 and male 25), and third group included another 50 apparently healthy volunteers (female 20 and male 30). The patients were admitted from the National Center of Hematology/Mustansiriyah University. All patients are diagnosed according to a complete blood count (CBC), a bone marrow examination, and a BCR-ABL gene test.

RESULTS: Reverse transcription-quantitative polymerase chain reaction was applied to assess the expression levels of the *HIF-1A* gene and serum level of MCP1 by enzyme-linked immunosorbent assay. The results displayed downregulated of the *HIF1A* gene messenger RNA in CML patients in comparison to the controls group, as well as no statistically significant link was discovered when the fold of expression was correlated with the age and gender of CML patients.

CONCLUSION: *HIF1-alpha* gene has an important role in pathological pathways such as angiogenesis. According to this study, *HIF1-alpha* gene is not an appropriate prognostic biomarker for detecting the risk of CML as well as MCP1 is thought to be a predictor of CML progression.

Keywords:

Chronic myeloid leukemia, hypoxia-inducible factor-1a, monocyte chemoattractant protein-1

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Introduction

Chronic myeloid leukemia (CML) is a hematopoietic stem cell disease.[1] Most chronic phase CML patients experience a full hematologic and cytogenetic response when treated with tyrosine kinase inhibitors (TKIs).[2] There are three stages to CML: chronic phase (CP), which if untreated progresses to the accelerated phase (AP), then the blast phase (BP). However, the use of TKIs as a treatment has slowed the disease's progression to blast

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crisis.[3] Increased leukemic stem cells' genomic instability frequently coincides with the progression of CML from the CP into the BP.[4,5] Blood cell transformation is caused by the accumulation of genetic and epigenetic variations in the genes that control these pathways, which leads to the destruction of the mechanism that controls the typical cell division process, the blocking of cellular differentiation, the loss of response to the mechanism of programmed death, and the self-renewal of transformed blood cells.[6] Leukemia development and regulation are influenced by chromosomal abnormalities and gene mutations.[7]

Hypoxia-inducible factors (*HIFs*), heterodimeric transcription factors that in the end, control cellular reactions to changes in oxygen tension during normal development or pathological processes, such as cancer or cardiovascular disease, are among the most frequently altered genes in CML patients.[8] The two subunits that comprise *HIF1* are α and β.[9] *HIF1A*'s gene is located on chromosome 14 (14q21-q24).[10] The level of *HIF1A* protein is linked to the concentration of oxygen in cells; under hypoxemic circumstances, *HIF1A* does not break down and instead builds up in the cell nucleus.[11] *HIF1A* stimulates the processes of angiogenesis, glycolysis, erythropoiesis, and vasodilation.[12]

HIF1A and its messenger RNA (mRNA) have a very short half-life of 5 min in normoxic circumstances.[13] In many malignancies in humans, *HIF1A* is overexpressed. Because *HIF1A* overexpression triggers angiogenesis and controls cellular metabolism to overcome hypoxia, it plays a significant role in promoting the growth and spread of tumors.[14]

Immune system modulators, including chemokines and cytokines, are important in CML.[15,16] When an injury or inflammatory state resolves, pro- and anti-inflammatory cytokines are released, which regulate cellular stress and tissue damage. Cytokines then return to homeostatic levels.[17] Monocyte chemoattractant protein-1 (MCP-1) gene is located at chromosome 17q11.2-q21.1. It consists of three exons and two introns. MCP-1 is also recognized as chemokine ligand CCL2.[18] MCP1 controls monocyte/macrophage migration and infiltration. Monocyte migration from the bloodstream across the vascular endothelium is necessary for both the immune system's regular monitoring of tissues and its reaction to inflammation.[16,19] Frequently released by tumor cells, fibroblasts, macrophages, and endothelial cells.[20] Because they all have the cognitive receptor CCR2 on them, monocytes, dendritic cells, memory T-cells, and basophils are among the various cell types that are drawn to the chemokine CCL2. Numerous studies demonstrate that malignant cells of hematological and solid tumors express high levels of CCL2.[19] Chronic inflammation is caused by abnormal cytokine production and dysregulation of cytokine levels, which is linked to the pathophysiology of several diseases, including cancer.[21]

All forms of leukemia are characterized by cytokine and growth factor signaling, which supports leukemia growth, survival, self-renewal, and chemotherapy resistance.[22]

There are many studies in Iraq on predictive biomarker for CML progression and development.[23-26]

This study's objective was designed to investigate the role of *HIF1A* gene expression with CML, as well as to evaluate the role of MCP-1 as a predictive biomarker on disease progression.

Patients, Materials and methods

This study consists of three groups with CML. The patients' ages ranged from 35 to 62 years: the first group includes 50 newly diagnosed CML patients (males 28 and females 22), the second group consists of fifty CML patients treated with TKI with the complete molecular response (p210 *BCR-ABL* transcript levels ≤0.1% IS) (male 25 and female 25), and the third group includes another 50 apparently healthy volunteers (male 30 and female 20). Personal information such as age, sex, duration of disease, and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) result for BCR-ABL1 IS (%) were also included. All patients are diagnosed according to a complete blood count (CBC), a bone marrow examination, and a BCR-ABL gene test.

The sample of pateints and control were selected from the National Center of Hematology/Mustansiriyah-University; this study was conducted in Baghdad from March 2023 to November 2023.

The study was approved by review ethical committee of Institute of Genetic Engineering and Biotechnology for Postgraduate Studies/University of Baghdad. Written informed consents were obtained from all patients and apparently healthy control group.

Patient's exclusion criteria

Patient's age <18 years' old, patient suffering from liver or kidney disease those who suffer from hepatitis B and C, and patients with (HIV) were also excluded from the study.

Blood sample collection

Blood sample 5 mL was collected from each individual in each group, 3 mL blood was kept in ethylenediaminetetraacetic acid tubes for CBC, 250 µL

blood from the 3 mL was added to 750 µL *TransZol* Up in Eppendorf tube to estimate the *HIF1A* gene expression by quantitative real-time PCR (qRT-PCR), as well as 2 mL for enzyme-linked immunosorbent assay (ELISA).

RNA extraction

RNA was extracted from samples using the protocol *TransZol Up* Plus RNA Kit (Transgen, China). Then, RNA purity and concentration were determined by NanoDrop.[27] The acceptable purity of RNA was 2.025 ± 0.05 in CML patients, and for the control group was 2.058 ± 0.053, the concentration of RNA samples (ng/µL) in controls and CML patient's groups was (98 ± 23.52) and (105 ± 35.73), respectively.

For determining the level of MCP-1 in serum of CML patients and apparently healthy by using ELISA kit (SUNLONGBIOTECH/ China).

cDNA synthesis for messenger RNA

The cDNA synthesis was subjected by using the protocol in EasyScript® One-Step gDNA Removal and cDNA Synthesis SuperMix (Catalog No. AE311). The steps wase shown in Table 1.

TransStart® top Green qPCR Super Mix as directed by the manufacturer; this process was performed in a reaction volume of 20 µl. qRT-PCR Calculating the *HIF1A* gene's expression levels was done by qRT-PCR. To verify that the target gene is expressed, a quantitative real-time qRT-PCR SYBR Green assay was used. Table 2 presents the primer sequences. The mRNA levels of endogenous control (housekeeping gene) glyceraldehyde-3-phosphate dehydrogenase (*GAPDH)* were prepared using the sequence of gene primers, amplified, and used to normalize the housekeeping mRNA levels.[28]

QRT-PCR was conducted using the Qiagen Rotor-Gene real-time PCR system and qPCR soft software.

Table 1: Thermal cycler steps for cDNA reverse transcription conditions

	Step 1	Step 2	Step 3
Temperature (°C)	25	42	85
Time	10 min	15 min	5 s

The cycle threshold (Ct) was used to measure the fold change and gene expression levels using the components of the TransStart® Top Green qPCR SuperMix Kits.

Relative gene expression measurement using quantitative

Real-time reverse transcription-quantitative-polymerase chain reaction

The levels of expression (*HIF-1)* gene and housekeeping gene *GAPDH* were calculated by RT-PCR to verify the target gene's expression. Table 3 describes the components of the reaction.

Gene expression calculation

The degree of gene expression fold is the ratio that depends on the presence of the calibrator value and the mean ΔCt of the patients and mean ΔCt of the control and all the calculations cannot be done in the absence of the housekeeping gene values.[29]

Statistical analysis

The results used one-way ANOVA. If the $P \leq 0.05$, statistical significance was taken into account. Using Pearson correlation to determine the correlation between two quantitative variables, receiver operating characteristic (ROC).

Results

Molecular examination

An extract of transcriptome RNA from the blood samples

From every sample, total RNA was successfully extracted. The concentrations and purity of extracted RNA are shown in Table 4.

Expression of gene

Gene expression was assessed in the present investigation using RT-qPCR, a fluorescent dye capable of identifying any type of double-strand DNA, including cDNA and a Ct value representing the amplification was recorded. A lower Ct value suggested that the target was present in greater quantities and vice versa.

A low Ct value denotes high gene expression, and the highest value denotes low gene expression.

Table 2: The primers employed in the research

Primer	Sequence from 5'–3' direction	Primer size (bp)	Product size (bp)	Temperature (°C)
HIF1A (gene expression)				
Forward	GAAAGCGCAAGTCCTCAAAG	20	167	60
Reverse	TGGGTAGGAGATGGAGATGC	20		
GAPDH (Primer Bank ID NO. 378404907c2)				
Forward	ACAACTTTGGTATCGTGGAAGG	22	101	64
Reverse	GCCATCACGCCACAGTTTC	19		

GAPDH=Glyceraldehyde-3-phosphat dehydrogenase, HIF1A=Hypoxia-inducible factor 1A, BP=Blast phase

Validation of reference gene in study groups

At the level of a housekeeping gene, the gene expression was normalized. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and quantified by the ΔCt value and folding (2-ΔCt) method.

Comparison between the mean Ct of the controls, chronic phase (CP), and newly diagnosed CML patients' group revealed there is not a noticeable difference between these groups ($P > 0.05$). The mean Ct ±SD values of *GAPDH* in these groups were as follow: 13.683±33297, 13.672±37669, 13.766±21548 respectively was shown in Table 5 a apparently healthy controls, b CML: Chronic phase patients, c CML patients newly diagnosed was no significant between them. The expression fold for *GAPDH* gene was shown in Table 6.

The use of reference genes in molecular investigations is based on the hypothesis that their expression stays stable in the cells or tissues under study.[30] The *GAPDH* gene is one of the most frequently employed reference genes in gene expression studies.[31]

The *GAPDH* gene amplification plots are depicted in Figure 1.

Real-time polymerase chain reaction quantification of hypoxia-inducible factor 1a expression

The amplification plots and dissociation curves for the *HIF1A* gene are shown in Figure 2a and b. Using the 2-ΔCt method, in Table 7 a Controls: Apparently healthy controls, b CML patients Chronic phase, c CML patients newly diagnosed and Figure 3 summarizes the level of expression of HIF1A gene mRNA in both the controls group and the CML patient's groups. The control group was with a mean Ct value of *GAPDH* of 13.683, whereas the patient's group had mean Ct values of 13.672 and 13.766, respectively. The normalized Ct values of *HIF1A* to the *GAPDH* (ΔCt) were 4.92 in control group, whereas in CML patient's groups, 8.793 in chronic phase CML and 7.614 in newly diagnosed CML. The expression level of *HIF1A* gene mRNA was downregulated (decreased) in patients with CML relative to the control group using a fold change (0.069) in chronic phase CML (0.1545) in newly diagnosed CML versus a fold change (1) in the control group.

Correlation of gene expression folding of hypoxia-inducible factor 1a gene with gender in chronic myeloid leukemia patients

No statistically significant variation was observed in the mean gene expression folding for the *HIF1A* gene in the female patient group and the male patient group with CML as shown in Table 8.

Table 3: The thermal profile of glyceraldehyde-3-phosphate dehydrogenase and hypoxia-inducible factor 1A gene expression

Step	Temperature (°C)	Time (s)	Cycles
Enzyme activation	94	30	1
Denaturation	94	5	40
Annealing*	58–56	15	
Extension	72	20	
Dissociation	55–95		1

*Ta: Annealing temperature, for HIF1A was 56°C and for GAPDH was 58°C. GAPDH=Glyceraldehyde-3-phosphate dehydrogenase, HIF1A=Hypoxia-inducible factor 1A

Table 4: Concentrations and purity of extracted RNA

Group	Total RNA concentration (ng/μL), mean±SD	*P*
Controls[a]	98±23.52	0.6
CML patients[b]	105±35.73	
Purity (260/280)		
Controls[a]	2.058±0.053	0.6
CML patients	2.025±0.052.025±0.05	

[a]Apparently healthy controls. CML=Chronic myeloid leukemia, SD=Standard deviation

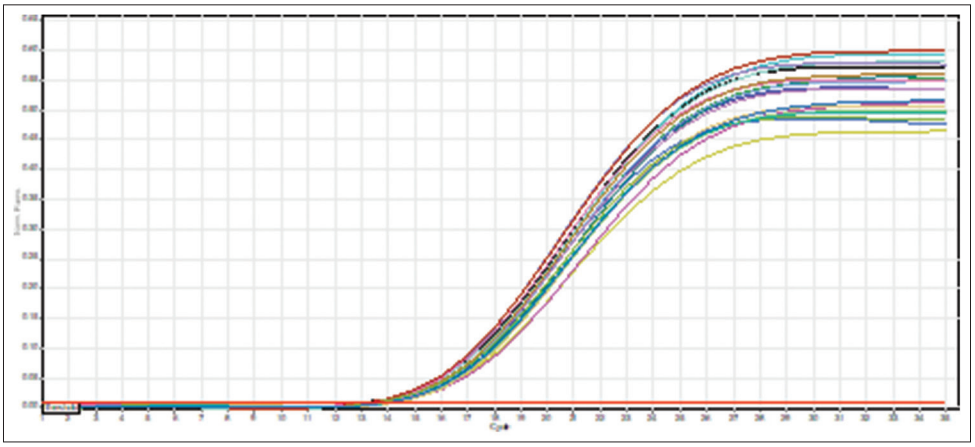

Figure 1: Glyceraldehyde-3-phosphate dehydrogenase gene every study group was represented in the amplification plots by quantitative polymerase chain reaction (qPCR) samples. The Qiagen Rotor-Gene Q 6000 qPCR machine was used to take the picture directly

Table 5: Comparison of the mean Ct values of the reference gene glyceraldehyde-3-phosphate dehydrogenase among controls (chronic phase) patients, and (newly diagnosed) chronic myeloid leukemia patients groups

Group	*n*	Mean Ct±SD	SE	Range	*P*	Significance
Controls[a]	50	13.683±33,297	0.04066	13.17–14.03	0.8	NS
CP patients[b]	50	13.672±37,669	0.05073	13.25–14.32		
Newly diagnose patients[c]	50	13.766±21,548	0.02753	13.50–14.03		

[a]Apparently healthy controls, [b]CML: Chronic phase patients, [c]CML patients newly diagnosed. SD=Standard deviation, NS=Not significant, CP=Chronic phase, SE=Standard error

Table 6: Comparison between study groups regarding reference gene glyceraldehyde-3-phosphate dehydrogenase fold expression

Group	Mean Ct of GAPDH	2^{-Ct}	Experimental group/control group	Fold of gene expression
Controls[a]	13.683	0.000076	0.000076/0.000076	1+
CML (CP)[b]	13.672	0.000077	0.000077/0.000076	1.007731
CML newly diagnose[d]	13.766	0.000072	0.000072/0.000076	0.944311

[a]Apparently healthy controls, [b]CML: CP patients, [c]CML patients newly diagnosed. CP=Chronic phase, GAPDH=Glyceraldehyde-3-phosphate dehydrogenase, CML=Chronic myeloid leukemia

Table 7: Using the $2^{-\Delta Ct}$ method, the expression level of hypoxia-inducible factor 1a gene mRNA was determined in control and CML patient groups

Groups	Means Ct of HIF1A	Means Ct of GAPDH	ΔCt (means Ct of HIF1A)	$2^{-\Delta Ct}$	Experimental group/ control group	Fold of gene expression
Control	19.773	13.683	4.92	0.0330	0.0330/0.0330	1.00
Chronic	22.465	13.672	8.793	0.0023	0.0023/0.0330	0.069
New	21.38	13.766	7.614	0.0051	0.0051/0.0330	0.1545

[a]Controls: Apparently healthy controls, [b]CML: CP patients CHP, [c]CML patients newly diagnosed. CP=Chronic phase, GAPDH=Glyceraldehyde-3-phosphate dehydrogenase, CP=Chronic phase, HIF1A=Hypoxia-inducible factor 1A

Table 8: Gender and the folding of the hypoxia-inducible factor 1a gene expression correlation in chronic phase patients

Groups	Sex	Mean	SEM	*P*
Control	Male	0.9658	0.22219	0.8
	Female	1.0166	0.28650	
CP	Male	0.3883	0.08901	0.8
	Female	0.3691	0.09034	
New	Male	0.4243	0.12399	0.7
	Female	0.4998	0.15401	

SEM=Standard error of mean, CP=Chronic phase

Table 9: Correlation of gene expression folding of hypoxia-inducible factor 1a gene with age in chronic phase patients

	Age	Fold HIF1A
Age		
Pearson correlation	1	0.074
Significant (two-tailed)		0.369
Fold HIF1A		
Pearson correlation		1
Significant (two-tailed)		

HIF1A=Hypoxia-inducible factor 1A

The average folding of the *HIF1A* gene in the female patient group and control group was (0.3691 ± 0.09034), (0.4998 ± 0.15401), and (1.0166 ± 0.28650) in chronic phase, newly diagnosed, and control group, respectively, compared to male for all groups, and the differences were statistically insignificant $P > 0.05$.

Correlation of gene expression folding of hypoxia-inducible factor 1a gene with age in chronic myeloid leukemia patients and apparently healthy

No statistically significant distinction could be found between gene expression folding for the *HIF1A* gene and age for all groups as shown in Table 9.

The correlations between hypoxia-inducible factor 1a expression and clinical marker hematology

This result showed there was a highly significant correlation between white blood cell (WBC) and platelets and also a high significant between platelets and hemoglobin, whereas there was no significant correlation between the *HIF1A* gene and all hematological markers. The result is shown in Table 10.

Serum level of human monocyte chemotactic protein 1 by enzyme-linked immunosorbent assay

The results of the concentration of the serum level of MCP1 are shown in Table 11.

MCP1 (pg/ml) levels showed high significant differences among patients (newly diagnosed) compare to Chronic phase and control in the current study this result was shown in [Figure 4].

The receiver operating characteristic curve

The ROC analysis can discriminate between two patient states, typically referred to as "diseased" and

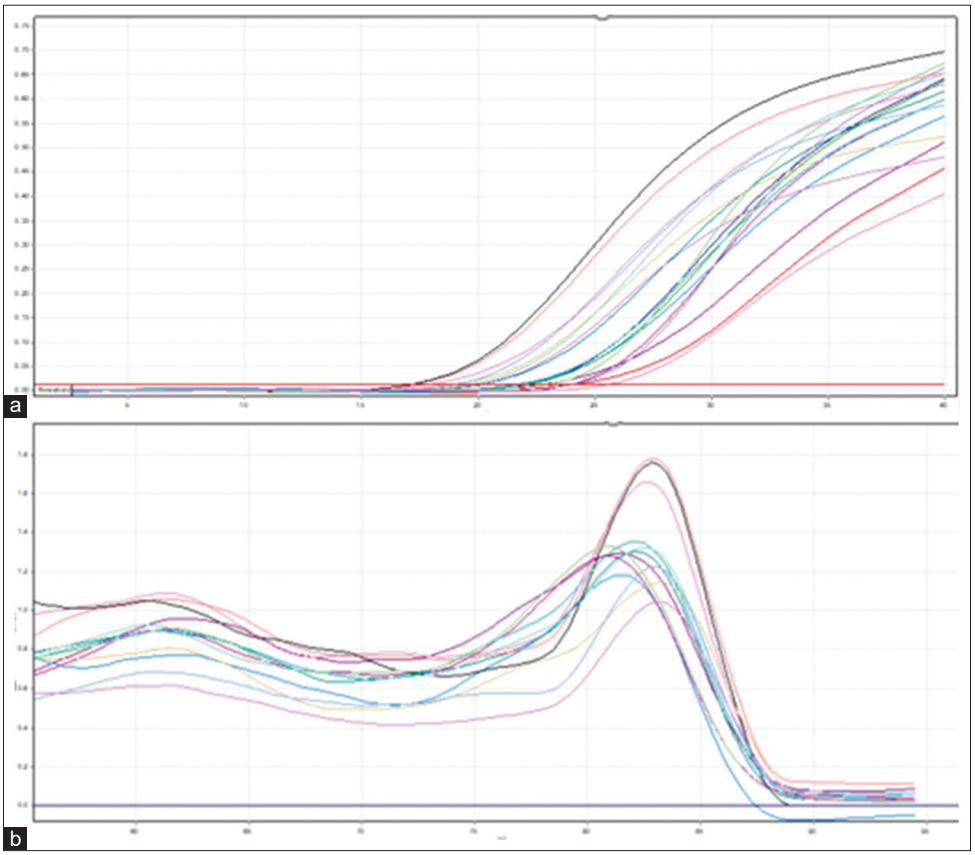

Figure 2: (a) Amplification plot of hypoxia-inducible factor 1 a (*HIF1A)* gene, (b) melting curve of *HIF1A* gene expression

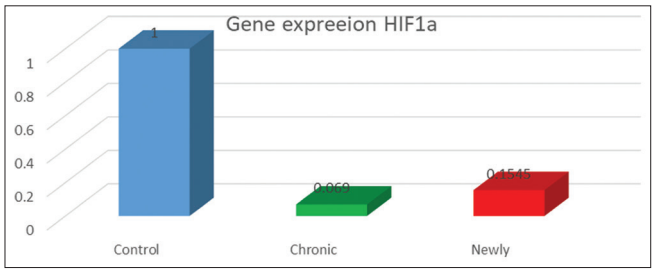

Figure 3: The differences between the CML patient (chronic phase), (newly diagnosed), and apparently healthy control according to hypoxia-inducible factor 1 a gene expression. HIF 1a = Hypoxia-inducible factor 1 a

"nondiseased," so it may be used in clinical epidemiology to quantify how accurately medical diagnostic tests.[32] The area under the curve is an effective and combined measure of sensitivity and specificity that describes the inherent validity of diagnostic tests; the X-axis or independent variable is the false-positive rate for the predictive test. The Y-axis or dependent variable is the true positive rate for the predictive test. Each point in ROC space is a true-positive/false-positive data pair for a discrimination cutoff value of the predictive test.[33]

Results in table 12 and Figure 5, demonstrated that the threshold between sensitivity (98) and specificity (73) for MCP1 was 348.

Table 10: The correlations between hypoxia-inducible factor 1a expression and clinical marker hematology

Fold HIF1A	Fold HIF1A	WBC	HP	PLT
Pearson correlation	1	0.156	0.129	−0.117
Significant (two-tailed)		0.056	0.117	0.153
WBC				
Pearson correlation		1	−0.164*	0.347**
Significant (two-tailed)			0.045	0.000
HP				
Pearson Correlation			1	0.400**
Significant (two-tailed)				0.000
PLT				
Pearson Correlation				1
Significant (two-tailed)				

*Significant, **High significant. HIF1A=Hypoxia-inducible factor 1a, PLT=Platelets, HP=Hemoglobin, WBC=White blood cell

Table 11: Serum level of human monocyte chemotactic protein 1

Collectives	Mean	SD	SEM	*P*
Control	277.395816[c]	66.3225985	9.4746569	0.001**
CP	348.105784[b]	101.8027009	14.2552295	
New	414.138750[a]	46.7052254	6.6051163	

**high significant, a significant than b, b significant than c, a significant than b and c . CP: Chronic phase, SD=Standard deviation, SEM=Standard error of mean

The result revealed that the area under the curve for MCP1 was 0.85 and gives high significant differences

Table 12: Receiver operating characteristic curve data of the studied gene

Parameters	AUC	Explanation	*P*	The best cut off	Sensitivity (%)	Specificity (%)
MCP1	0.85	Very good	0.001	348	98	73

AUC=Area under the curve, MCP1=Monocyte chemoattractant protein-1

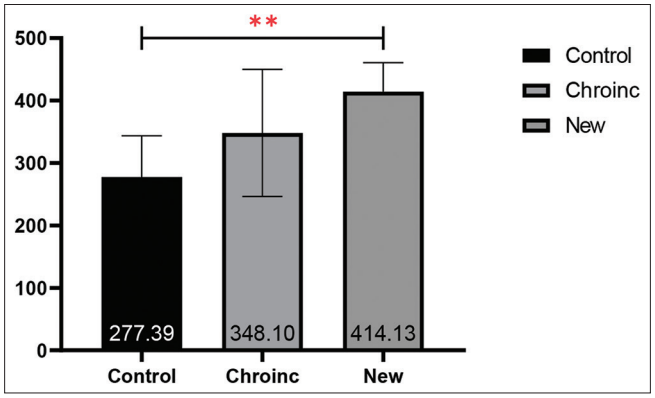

Figure 4: The serum level of monocyte chemoattractant protein 1 in patient's groups compare with apparently healthy control. MCP 1 = Monocyte chemoattractant protein 1

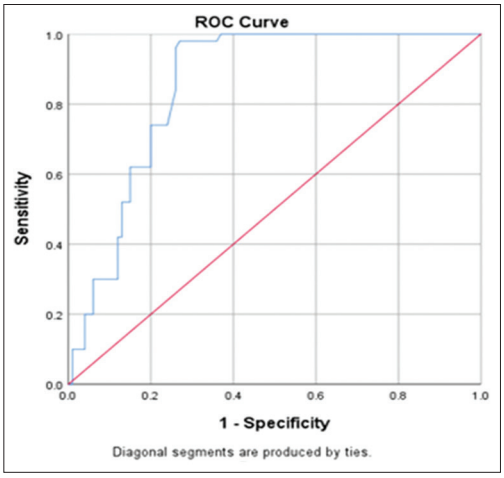

Figure 5: Receiver operating characteristic curve of the monocyte chemoattractant protein-1. ROC = Receiver operating characteristic

$P \leq 0.01$. When the value of the area under the curve equals 0.001 or higher this mean, it is perfect in anticipating disease prognosis.

Discussion

HIF-1A gene located on chromosome 14.[34] As a master regulator of the systemic and cellular homeostatic response to hypoxia, *HIF-1* activates the transcription of numerous genes, including those related to angiogenesis, apoptosis, and energy metabolism. Increased *HIF* expression has been linked to a number of malignancies.[35] According to this study, the expression level of *HIF1A* gene mRNA was downregulated, there was no significant difference in HIF1-alpha mRNA expression between the CML patient group and the control group.

HIF-1 alpha gene has a critical role in angiogenesis and tumor malignancy, but in this study, we did not find significant expression of *HIF-1* alpha mRNA in CML patients; this result is similar to the study with colorectal polyps that suggest *HIF-1 alpha* gene expression is not a suitable biomarker for evaluating malignancy risk in colorectal polyps.[36] discovered that *HIF-1* alpha was expressed immune histologically in hyperplastic polyps, but this expression was not significant, and these findings are consistent with this finding.[37] Researchers mentioned that *HIF1-alpha* expression was not observed in CML and upregulation of *HIF1-alpha* in carcinoma may have arisen from genetic alteration and genome instability.[38] In our study, *HIF1A* downregulation was detected. One could identify two scenarios to reconcile these disparities: First, there is evidence that *HIF1A* functions as a tumor suppressor gene. For example, in murine AML, a loss-of-function mutant of *HIF1A* can cause accelerated leukemogenesis by unintentionally activating certain oncogenes. Second, the gene that codes for *HIF2A* shares a high degree of sequence homology with *HIF1A*, suggesting that it may be involved in angiogenesis, proliferation, and glucose metabolism during prolonged hypoxic conditions.[39] Researchers mentioned that *HIF1-alpha* expression was underexpressed when compared to the control.[40] According to a different study, distinct subtypes of leukemia may have distinct functions for *HIF1A* mRNA expression.[41]

The mean age of CML patients in Iraq at diagnosis was 40.39 ± 14.30, which is younger than the age that has been reported globally for initial diagnosis, based on age group and *HIF1A* gene expression did not significantly correlate in CML patients and control.[42] Thus, based on the type of gene, we can infer that the association between patient gender and gene expression may differ. These results suggest that CML can damage individuals of any age, male or female (Our study revealed that high significant correlation between white blood cell, platelets and *HIF1A* expression). Ismail *et al*., in 2023, found CML patients had significantly higher WBC and platelet counts (28%) when compared to the responder CML patients.[43]

There were highly significant differences, according to immunological results between newly diagnosed CML patients and control group and significant differences between CML patient newly diagnosed compared to CML chronic phase. The goal of this study is to illustrate the traits of CML patients influence with incidence of the disease. This study was agreed with regard study to

chronic lymphocytic leukemia; MCP-1serum levels are high in CLL patients.[19] MCP-1/CCL2 chemokine was the most significantly increased in thymi from leukemic mice as its concentration, evaluated by ELISA, was increased by 5.4 fold compared with the control mice.[44] Before chemotherapy, CCL2 was present at very high levels. On the other hand, CCL2 dramatically dropped following the first round of chemotherapy, reaching a level similar to that of the healthy controls.[45]

Conclusion

HIF1-alpha gene has an important role in pathological pathways such as angiogenesis. According to this study, *HIF1-alpha* gene is not a suitable prognostic biomarker for detecting the risk of CML.

There was a significant correlation with WBC, while there was not any correlation between the rest of hematological markers and the progression of disease also, there was not a significant difference according to sex.

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Conflicts of interest
There are no conflicts of interest.

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