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Association of *HOXA4* Gene Expression and Methylation with Response to Treatment in Iraqi Chronic Myeloid Leukemia Patients

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Abstract: Genetic and epigenetic factors affect Chronic Myeloid Leukemia (CML) response to Imatinib mesylate (IM) therapy. This study aimed to investigate *HOXA4* gene methylation and expression in CML patients and their predictive value as response markers. Blood samples were collected from fifty CML patients (25 responders and 25 non-responders to Imatinib mesylate therapy) and 50 healthy controls of same age and sex. *HOXA4* gene methylation and gene expression studies were conducted by quantitative PCR (qPCR). Results revealed that CML patients had significantly higher level of *HOXA4* gene demethylation and expression compared to controls group (p <0.001). The non-responders CML patients showed higher significant levels of *HOXA4* gene demethylation (p<0.001) and expression (p<0.05) compared to responders CML patients. Significant risk association results of *HOXA4* demethylation and expression levels (p<0.001 and p<0.05, respectively) with the development of IM resistance in CML patients according to optimal cut-off point obtained by receiver operating characteristics (ROC) analysis. In conclusion, *HOX*A4 gene activation due to promoter DNA hypomethylation refers to its oncogenic role in CML pathogenesis. *HOXA4* gene demethylation and overexpression may serve as biomarkers for predicting IM resistance in CML patients, especially *HOXA4* gene demethylation with good sensitivity and specificity.

Key words: Chronic Myeloid Leukemia, *HOXA4* gene, response to treatment, Imatinib mesylate.

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Introduction

Leukemia ranked fifth among the top ten most prevalent malignancies in Iraq, according to the annual report of the Iraqi cancer registry for 2019 (1). Chronic myeloid leukemia (CML) is a myeloproliferative malignancy characterized by hematopoietic stem cell neoplastic transformation, leukocytosis, splenomegaly, and the pathognomonic presence of the Philadelphia (Ph) chromosome, t (9;22) (q34;q11), which results from the translocation of the proto-oncogene Abelson murine leukemia (*ABL1*) gene, located on chromosome 9 q34, to chromosome 22 q11, where the breakpoint cluster gene (*BCR*) is located. The molecular consequence of this translocation is the generation of a *BCR-ABL1* fusion oncogene, which in turn translates into a BCR-ABL1 oncoprotein, a constitutively active tyrosine kinase that causes CML and is the target of tyrosine kinase inhibitors (TKIs) (2). Chronic myeloid leukemia is a three-phase disease, with 85% - 90 % of patients initially presenting in a chronic stable phase and the remaining presenting in an accelerated phase or blast crisis. Without treatment, chronic phase CML proceeds inexorably to

accelerated phase/blast crisis, however, treatment with tyrosine kinase inhibitors (TKIs) has drastically lowered the pace of progression to blast crisis (3). Treatment resistance in malignant diseases, including leukemia, can emerge as a result of genetic and epigenetic changes in patients. Mutations in the drug's molecular target, extensive cellular changes, alterations in the way the drug interacts with the tumor, anomalies in the tumor microenvironment and remodeling of cancer cells epigenomic landscapes are examples of molecular changes that contribute to intrinsic or acquired treatment resistance (4-6). Despite of the remarkable efficacy of TKIs, a proportion of CML patients will acquire resistance. Both genetic and epigenetic factors influence the effectiveness of CML therapy. Resistance mechanisms involving *BCR-ABL* dependen pathways and *BCR-ABL* independent pathways have been categorized as the two most common mechanisms of TKIs resistance (7). *BCR-ABL* dependent pathways, comprising multiple *BCR-ABL* mutations, that have been identified as the most prevalent cause of IM resistance. Nonetheless, the reason of IM resistance in CML patients without *BCR-ABL* mutations may be attributed to aberrant *BCR-ABL*-independent mechanisms involving alternate signaling or epigenetic pathways. In addition to BCRABL1 protein, several other proteins such as transcription factors, transporter protein, and a great number of other proteins are crucial for regulating the high rate of proliferation and the suppression of apoptosis (7-9).The Homeobox (*HOX*) genes produce a highly conserved family of transcription factors. In humans, four *HOX* gene clusters, *HOXA*, *HOXB*, *HOXC*, and *HOXD*, are located on different chromosomes .The *HOX* gene networks function as master regulatory transcription factors that are critical regulators of embryonic development, hematopoietic differentiation, and play an important role in development of hematological malignancies, treatment response, and prognosis (10).The aim of this study was to investigate the correlation of methylation and gene expression pattern of *HOXA4* with Imatinib mesylate (IM) response in Iraqi CML patients (responding and non-responding to Imatinib mesylate treatment) in comparison to controls, and evaluate the prognostic utility of them as markers for response to treatment.

Materials and methods

Study subjects

This is a case-control study which was conducted through the period from March 2021 to August 2022. The study protocol was approved by the Ethics Committee of the Iraqi Ministry of Health and Environment, and a written informed consent was obtained from all participants before entering the study. Fifty cases of CML patients (25 responders CML patients and 25 non-responders CML patients to Imatinib mesylate therapy) on Imatinib Mesylate (IM) therapy for at least one year as frontline therapy were collected from Baghdad Teaching Hospital/ Medical City and The National Center of Hematology/ Mustansiriyah University, and fifty subjects as an apparently healthy individuals with similar age and sex, were recruited as controls group. Patient's response to IM based on molecular and hematological response results according to European Leukemia Net 2020 (11). From each study subject, patients and controls volunteers, peripheral blood (PB) (5 ml)

was collected, dispensed in 2 tubes containing k3EDTA each with 2 ml, one for CBC while another one for DNA and RNA extraction.

Total RNA and genomic DNA extraction

Total RNA was extracted from 0.25 ml k3EDTA blood homogenized with 0.75ml Tri reagent (Zymo Research, USA) according to company protocol with some modification. DNA extraction was done using ReliaPrep™ Blood gDNA Miniprep System (Promega, USA) according to the manufacturer's guidelines. The concentration and purity of the purified RNA and DNA were measured by NanoDrop, Q5000 (Quawell, USA) microvolume UV-Vis spectrophotometer.

Methylation of *HOXA4* gene

Genomic DNA was modified with sodium bisulfite using the Qiagen EpiTect Fast DNA Bisulfite Kit (Qiagen, Germany). Cleanup of converted DNA was done according to the manufacturer's protocol. Methylation-specific quantitative PCR (MS-qPCR) of *HOXA4* gene was done by TransStart Tip Green qPCR Super Mix kit (TransGen Biotech Co, China) and the two pairs of primers specific for the methylated (M) and unmethylated (U) *HOXA4* promoter regions , designed using MethPrimer software, in separate reactions for each sample . Using the Rotor-Gene Q (Qiagen, Germany) used to detect the fluorescence, with software version 2.3.1.49., 0.5 µl of each forward and reverse methylated /Unmethylated primer (10µM) and 2 µl of 50–100 ng of bisulfite-converted genomic DNA (bcDNA), real-time PCR was done in a final reaction volume of 20 µl. In each experimental run full methylated bisulfite-converted DNA (Epitect PCR control DNA set, Qiagen, Germany) served as the positive control for methylated primers, while unmethylated bisulfite-converted DNA served as the negative control for both methylated and unmethylated primers. Methylated and unmethylated *HOXA4* primers sequences are listed in table (1). The thermal profile was as follows: hold at 94 C° for 60 seconds (1 cycle), then 40 cycle: denaturation at 94 C° for 5 seconds, annealing at 54 C° for 15 seconds, and extension at 72 C° for 20 seconds, finally dissociation from 65 C° to 95 C° (5 seconds for 1 degree). The demethylation rate (DMR %) of *HOXA4* was determined using the formula: $100/ [1 + 2^{(CtTG-CtCG)}] 100\%$ (12). The cycle threshold attained with TG (unmethylated) primers is denoted by the symbol CtTG in this formula, whereas CtCG denotes the cycle threshold attained with CG (methylated) primers.

***HOXA4* gene Expression**

Total RNA was reverse-transcribed to complementary DNA (cDNA) using the EasyScript One-Step gDNA removal and cDNA Synthesis SuperMix Kit (TransGen Biotech Co, China) in a reaction total volume of 20 µl, according to the manufacturer's instructions. Quantitative Real-Time PCR (qRT–PCR) for gene expression *of HOXA4* was done by using 10 µl of PerfectStart™ Green qPCR SuperMix kit (TransGen Biotech Co, China), 3 µl cDNA, 1 µl for each of forward and reverse primers (10µM) listed in table (1), and the Rotor-Gene® Q (Qiagen, Germany) was used to detect the fluorescence. *HOXA4* primers were designed by Primer 3 plus software. For each sample of study groups, reaction was done in duplicates, and a no template control (NTC), as a negative

control, was included in each run. The thermal profile was as follows: hold at 94 C° for 60 seconds (1 cycle) , then 40 cycle: denaturation at 94 C° for 5 seconds , annealing (at 56 C° for *HOXA4* and 58 C° for *GAPDH*) for 15 seconds , and extension at 72 C° for 20 seconds. Finally, the dissociation was from 65 C° to 95 C° (5 seconds for 1 degree). The specificity of the amplified product was verified by melting curve analysis. The expressions were quantified relative to the glyceraldehyde-3-phosphate dehydrogenase *(GAPDH*) reference gene using the $2^{-\Delta\Delta CT}$ method (13) to calculate the relative expression of *HOXA4* gene in the studied group samples. The data were expressed as the fold change in *HOXA4* gene expression in study groups (CML patients, responders and non-responders CML patients) relative to healthy controls and normalized to the expression levels of the reference gene (*GAPDH*). Statistical significant differences in the levels of mRNA expression *of HOXA4* gene was assessed according to the median fold of expression level of *HOXA4* in study groups.

Table (1): Primers for *HOXA4* gene methylation and gene expression study.

Gene expression primers		
Primer	Sequence (5′ →3′ direction)	Product size
HOXA4		
Forward	ACCAAGATGCGATCCTCCAA	196
Reverse	GAACGGAGCAGGAGAAGAGA	
GAPDH **(PrimerBank ID NO. 378404907c2)**		
Forward	ACAACTTTGGTATCGTGGAAGG	101
Reverse	GCCATCACGCCACAGTTTC	
Methylation study primers		
Primer	**Sequence (5′ →3′ direction)**	**Product size**
HOXA4		
Methylated Forward	TTTATTGTTTTTTATTACGCGTCGC	176
Methylated Reverse	GCTAAACCCTTAACTTACGCCGAA	
Un-methylated Forward	GTTTATTGTTTTTTATTATGTGTTGTGG	178
Un-methylated Reverse	CACTAAACCCTTAACTTACACCAAA	

Statistical analysis

The Statistical Packages for Social Sciences-version 22 (SPSS-version 22) was used to analyze data. Normality test (Shapiro-Wilk test) was used to determine whether the studied quantitative parameters followed a normal distribution. Data were presented in simple measures of frequency, percentage and median (interquartile range, IQR). Parametric (independent sample t- test) and non-parametric (Mann-Whitney U-test) were used to compare between quantitative data of two groups. Qualitative data were presented as the frequency and percentage, and significant differences between their distributions in study groups were assessed by Pearson's Chi-squared or Fisher's exact tests, when appropriate. Receiver operator curve (ROC) analysis was used to determine an optimal cut-off point of *HOXA4* demethylation rate (DMR%) and gene expression level in recognizing non-responders from responders CML

patients. Later, the numerical data of *HOXA4* demethylation rate and gene expression level were divided into two groups according to the cut-off point calculated, to identify the risk of developing IM resistance. The odd ratios (ORs) and its 95% confidence interval (CI) were estimated. A probability (p) value $\leq$ 0.05 was considered as statistically significant.

Results and discussion

General characteristics of the studied groups

The studied subjects included 50 patients with CML receiving Imatinib mesylate therapy, 25 responders CML patients and 25 non-responders CML patients to Imatinib mesylate therapy, with a median (inter quartile range, IQR) treatment duration (month) of 48 (22.5-132) and 56 (22-96), respectively, without significant statistical differences (p>0.05). The effectiveness of TKIs in CML treatment was shown by many studies by improvement in 5-year survival, from 30 to 40% in the pre-TKI period to 96% following the drug's debut (14) Additional 50 apparently healthy controls with similar age and sex were recruited as controls group. Demographic, hematological and molecular characteristics of study groups listed in table (2). The median (IQR) age of patients group was 46.5 (38-54.5) while controls group was 46.50 (39-55) without significant differences (p>0.05). The median (IQR) age for responders and non-responders CML patients were 49(38-56.5) and 45 (38-54), respectively, without significant differences (p>0.05). This is comparable to Ning *et al*., 2020 review (15) as a younger age distribution among Asian population was younger than 50 years old compared to older than 50 years old in western countries, and it's almost same to other Iraqi studies (16-19). This study found that females had a higher significant (p<0.05) failure rate to respond to medication than males (64% vs. 36%), which may be attributed to different sex compliance and socioeconomic background of patients during Imatinib shortage. In contrast, several recent researchers have found that women are more likely than men to have better molecular responses (20).

The result of this study showed that CML patients had significant lower (p<0.001) hemoglobin levels in (56%) of patients compared to controls group, significant higher (p<0.05) WBC, and platelets counts in (14%) of patients compared to controls. Comparison between responders and non-responders CML patients revealed that non – response to treatment associated with a significant lower (p=0.001) hemoglobin levels in (80%) of non-responders CML patients, significant (p=0.01) higher WBC, and platelets counts in (28%) of them. Patients who lose their Imatinib response and progress to a more severe form of the disease experience bone marrow suppression attributable to an increase in cloned leukemic cells (21). The responders CML patients exhibited also a lower hemoglobin level in (32%) of them, which may be related to their long-term therapy with Imatinib (22). The median (IQR) of BCR-ABL % (IS) was significantly higher (p<0.001) for non-responders compared to responders CML patients, 0.84 (0.34-6.255) and 0.0032 (0.0001-0.0245), respectively. This difference served to highlight the characteristics of the patient groups that were examined in this study. These results defined the CML patients' response to TKI according to European Leukemia Net 2020 (11).

Table (2): Demographic, hematological and molecular characteristics of study groups.

Variables	Group		*p*-value	Group		*p*-value
	Controls (n=50)	CML Patients (n=50)		Responders CML patients (n=25)	Non-responders CML patients (n=25)	
Age #(Years)	46.50 (39-55)	46.5 (38-54.5)	0.95 NS	49 (38-56.5)	45 (38-54)	0.627 NS
Sex, n (%)						
Male	26(52%)	26(52%)	1NS	17(68%)	9(36%)	0.024*
Female	24 (48%)	24 (48%)		8 (32%)	16(64%)	
Hemoglobin (g/dL)						
≥12 (g/dL)	50 (100%)	22 (44%)	<0.001**	17 (68%)	5 (20%)	0.001**
< 12(g/dL)	0 (0%)	28 (56%)		8(32%)	20(80%)	
White blood cells ($\times10^3/mm^3$) , n(%)						
≤ 10 ($\times10^3/mm^3$)	50 (100%)	43 (86%)	0.012*	25(100%)	18 (72%)	0.01*
>10 ($\times10^3/mm^3$)	0 (0%)	7(14%)		0(0%)	7 (28%)	
Platelets ($\times10^3/mm^3$), n(%)						
<450 ($\times10^3/mm^3$)	50 (100%)	43 (86%)	0.012*	25(100%)	18 (72%)	0.01*
>450 ($\times10^3/mm^3$)	0 (0%)	7 (14%)		0(0%)	7 (28%)	
BCR-ABL %(IS)#	-	-	-	0.0032 (0.0001-0.025)	0.84 (0.34-6.255)	<0.001**
Treatment duration# (Months)	-	-	-	48 (22.5-132)	56 (22-96)	0.68NS

 # presented using their median (interquartile range, IQR) using Mann-Whitney U-test, NS: Non-significant. ,* and ** means significant at 0.05 and 0.01 levels respectively.

HOXA4 demethylation and expression

Demethylation rate of *HOXA4* gene was significantly higher ($p< 0.001$) in CML patients with a median (IQR) of 42.015 (27.034-69.709) compared to 4.816 (0.874-11.214) in controls group. The non-responders CML patients showed a significant higher demethylation rate of *HOXA4* ($p<0.001$) with a median (IQR) of 69.673 (46.296-77.440) compared to 28.882 (25.261-40.666) in responders CML patients. Table (3) and (4) list demethylation distribution in study groups and mRNA expression of *HOXA4* gene. The level of *HOXA4* gene mRNA was highly significant ($p<0.001$) in CML patients in comparison to controls group with a fold change (9.181) and the median (IQR) fold of *HOXA4* gene expression was 8.219 (2.278-55.552). The comparison between CML patients groups according to response to

treatment revealed a significant (p<0.05) higher *HOXA4* mRNA level in non- responders CML patients with a fold change 19.663 and median (IQR) expression 25.688 (3.596-141.392) than in responders CML patients with a fold change (4.287) and median (IQR) expression 3.324 (1.529-23.006). Table (5) and (6) summarizes expression level of *HOXA4* gene mRNA in controls and CML patients groups by the $2^{-\Delta\Delta Ct}$ method.

Table (3): Demethylation rate (%) and mRNA expression of *HOXA4* gene distribution in controls and CML patients groups.

Group	*HOXA4* Demethylation rate (DMR%) Median (IQR)	*p* value	*HOXA4* mRNA expression level		*p* value
			Fold change	Median (IQR) Fold expression level	
Controls	4.816 (0.874-11.214)	<0.001**	1	0.560 (0.135-4.573	<0.001**
CML Patients	42.015 (27.034-69.709		9.181	8.219 (2.278-55.552)	

NS: Non- significant. ,* and ** means significant at 0.05 and 0.01 levels respectively.

Table (4): Demethylation rate (%) and mRNA expression of *HOXA4* gene distribution in responders and non-responders CML patients groups.

Group	*HOXA4* demethylation rate (%) Median (IQR)	*P* value	*HOXA4* mRNA expression level		*P* value
			Fold change	Median (IQR) Fold expression level	
Responders CML patients	28.882 (25.261-40.666)	<0.001**	4.287	3.324 (1.529-23.006)	0.021*
Non-Responders CML patients	69.673 (46.296-77.440)		19.663	25.688 (3.596-141.392)	

NS: Non- significant. ,* and ** means significant at 0.05 and 0.01 levels respectively.

Table (5): Expression level of *HOXA4* gene mRNA in controls and CML patients groups by the $2^{-\Delta\Delta Ct}$ method.

Group	Mean Ct *GAPDH*	Mean Ct *HOXA4*	Δ Ct	Δ Ct Calibrator	ΔΔCt	2^-ΔΔCt	Experimental / control group	Fold change	Median fold (IQR)	*p* value
Controls	21.662	30.265	8.603	13.268	-4.665	25.369	25.369/ 25.369	1	0.560 (0.135-4.573)	<0.001**
CML Patients	21.61	27.0146	5.4044	13.268	-7.8636	232.905	232.905/ 25.369	9.181	8.219 (2.278-55.552)	

NS: Non- significant. ,* and ** means significant at 0.05 and 0.01 levels respectively.

Table (6): Expression level of *HOXA4* gene mRNA in in responders and non-responders CML patients groups by the $2^{-\Delta\Delta Ct}$ method.

Group	Mean Ct *GAPDH*	Mean Ct *HOXA4*	Δ Ct	Δ Ct Calibrator	ΔΔCt	2^-ΔΔCt	Experimental / control group	Fold change	Median fold (IQR)	*p* value
Responders CML patients	21.499	28.003	6.503	13.268	-6.765	108.745	108.745/ 25.369	4.287	3.324 (1.529-23.006)	0.021 *
Non-Responders CML patients	21.598	26.026	4.306	13.268	-8.962	498.828	498.828 /25.369	19.663	25.688 (3.596-141.392)	

NS: Non- significant. ,* and ** means significant at 0.05 and 0.01 levels respectively.

A receiver operating characteristic (ROC) curve analysis was used for the evaluation of the diagnostic value of *HOXA4* demethylation rate and gene expression for predicting response to treatment in CML patients (Table 7 and Figure1). A good and poor prediction of area under the curve (AUC) value results were seen for *HOXA4* demethylation rate % and gene expression with (p<0.001) and (p<0.05), at 0.87 and 0.69, respectively, among non-responders CML patients when compared to responders CML patients which indicated as markers, especially for *HOXA4* demethylation rate, to discriminate non-responders CML patients form responders CML patients. Accordingly, *HOXA4* demethylation rate (%) showed 80% positive predicative value and negative predicative value with 80% sensitivity and specificity at cutoff value more than 42.015%, while the *HOXA4* fold of gene expression showed 66.7% of positive predicative value and 62.1% negative predicative value with 56% sensitivity and 72% specificity at cutoff value more than 16.192 to differentiating non-responders from responders CML patients. Table (8) summarize risk association of *HOXA4* demethylation and expression levels with the development of IM resistance in CML patients according to optimal cut-off point. Demethylation rate of *HOXA4* > 42.015 and *HOXA4* level of gene expression > 16.192 were found to be associated with a significantly higher risk for developing Imatinib resistance with odd ratio (95%CI , p value) of 16 (4 -63.98, p<0.001) and 3.27 (1.01-10.62 , p<0.05) respectively. In contrast, Demethylation rate of *HOXA4* < 42.015 and *HOXA4* level of gene expression <16.192 were found to be associated with a significantly decreased risk for developing Imatinib resistance with odd ratio (95%CI, p value) of 0.06 (0.02-0.25), p<0.001) and 0.31(0.09 to 0.99), p<0.05), respectively. The result of this study revealed a significant higher level of *HOXA4* gene demethylation and expression in CML patients compared to controls and the non-responders CML patients showed higher levels compared to responders CML patients. Risk association results of *HOXA4* demethylation and expression levels with the development of IM resistance in CML patients according to optimal cut-off point obtained by ROC analysis indicated that both, *HOXA4* demethylation and expression levels, may serve as biomarkers for predicting IM resistance, and *HOXA4* demethylation rate has a good sensitivity and specificity to

discriminate non-responders from responders CML patients.

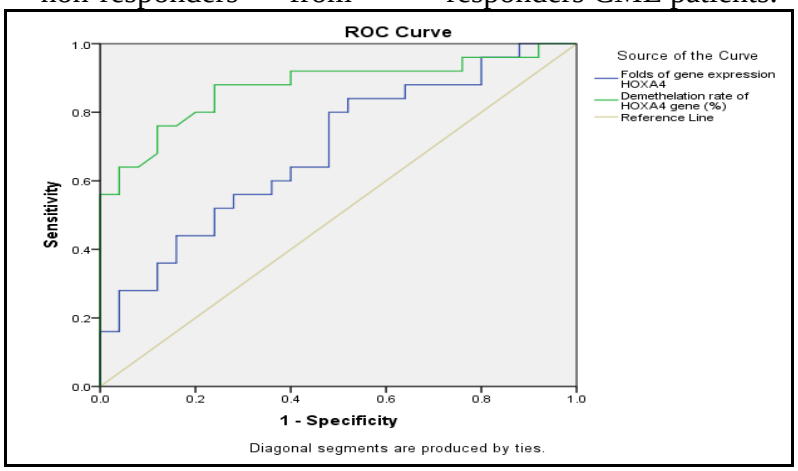

Figure (1): Receiver operating characteristics (ROC) curves of *HOXA4* demethylation rate (%) and fold of gene expression level among non-responders and responders CML patients.

Table (7): Optimal cut-off values of *HOXA4* demethylation rate (DMR%) and fold of gene expression for CML patients Imatinib resistance diagnostic and prognostic evaluation.

Parameter	AUC	AUC Explanation	95% CI Of AUC	AUC *p* value	Optimal Cut-off value	SN %	SP %	PPV %	NPV %
***HOXA4* gene DMR (%)**	0.87	Good	0.771-0.977	<0.001**	>42.015	80	80	80	80
***HOXA4* fold of gene expression**	0.69	Poor	0.532-0.828	0.021*	>16.192	56	72	66.7	62.1

DMR: Demethylation rate, AUC: Area Under the Curve, 95% CI of AUC: 95% confidence interval of area under the curve, SN: Sensitivity, SP: Specificity, PPV: Positive predicative value, NPV: Negative predicative value ,* and ** means significant at 0.05 and 0.01 levels , respectively.

Table (8): Risk association of *HOXA4* demethylation and expression levels with the development of IM resistance in CML patients.

Parameter	Responders CML patients n=25	Non-Responders CML patients n=25	OR (95%CI)#	X^2	*p* value
***HOXA4* gene Demethylation rate of (DMR%)**					
< 42.015	20 (80)	5(20%)	0.06 (0.02- 0.25)	18	<0.001**
>42.015	5(20%)	20(80%)	16 (4 -63.98)	18	<0.001**
***HOXA4* fold of gene expression**					
< 16.192	18 (72%)	11(44%)	0.31(0.09 to 0.99)	4.023	0.045*
>16.192	7 (28%)	14(66%)	4.023 (0.09- 0.99)	4.023	0.045*

#OR: Odd Ratio (95% confidence interval,* and ** means significant at 0.05 and 0.01 levels respectively.

There are 39 genes that constitute the *HOX* gene family. These genes encode homeodomain containing transcription factors that control development, blood cell differentiation, and the onset of leukemia (23**)**. Through regular hematopoiesis, *HOX* expression is silenced as hematopoietic cells

specialized. Hematopoietic precursors are unable to mature when their *HOX* genes are expressed in a non-native context, leading to fast self-renewal (24). In the normal adult hematopoietic system, The *HOXA* cluster is the most highly expressed. Among them, *HOXA4*, a transcription factor known to regulate haematopoiesis , was proven to produce mature myeloid and lymphoid progeny in hematopoietic stem cells and was shown to be important in the regulatory mechanisms of controlling hematopoiesis (25). Deregulation of *HOX* genes is a frequent contributor to leukemogenesis by affecting various pathways that promote leukemogensis including the activation of anti-apoptotic pathways, suppression of normal differentiation, and proliferation pattern (26,27). DNA methylation is involved in cell differentiation and helps to the maintenance of hematopoitic stem cell (HSC) stemness, as a result, the methylation profile varies depending on the cell type and differentiation stage (28) .Disruption of methylation pattern of the promoter considered an alternative to mutations in the coding sequences for tumor suppressor gene or oncogene that lead to disrupt their normal functions and is exhibited in a non-random way depending on the kind of tumor (29). Increased level of DNA methylation associated with transcriptional repression was observed in *HOXA4* gene in acute and chronic myeloid malignancies (30-32) and also reported in acute and chronic lymphoid leukemia (33, 34). Other Studies on Acute myeloid leukemia (35, 36) revealed elevated *HOXA4* expression level. These mentioned above results showed that *HOXA4* gene work as double agent, either tumor suppressor gene or oncogene depending on genetic and epigenetic modifications of the studied populations (37).

In conclusion, *HOX*A4 gene activation due to promoter DNA hypomethylation refers to its oncogenic role in CML pathgenisis , by interfering with the typical blood cell development, and mechanisms that influence therapeutic response. The role of hypomethylation and overexpression of *HOXA4* gene in resistance to Imatinib may be considered one of *BCR-ABL* independent resistance mechanisms and serve as biomarkers for predicting IM resistance, and *HOXA4* demethylation rate has a good sensitivity and specificity to discriminate correctly between CML patients according to their response to Imatinib.

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