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Variation In Expression Level Profile Of MicroRNA-150-1 As Prognosis Biomarker For Responsiveness Totyrosine Kinase Inhibitorsin Chronic Myeloid Leukemia Patients.

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ABSTRACT

Micro-ribonucleic acids (miRNAs) are small functional non-coding RNAs that regulate gene expression at the post-transcriptional level. Abnormal expression of specific miRNAs has been documented in chronic myeloid leukaemia (CML) and other types of leukemia and cancers. In CML, it was found that there is a correlation between responsiveness to Tyrosine Kinase Inhibitors(TKIs) and unregulated function of specific miRNAs. The aim of current cross-section study was to compare expression level profiles of miRNA-150-1between CML patients in different phases and had different degree of response to Imatinibmesylate (IM) and nilotinib(NIL). Peripheral blood samples (PB) from34 CML patients were collected. Those patients were grouped according to CML phase and responsiveness to IM based on hematological and molecular findings(at sampling) to: group-1 included 4 patients in accelerated phase (AP) and14 patients in blast crises (BC),whom considered as non-responders to IM and NIL. Group-2 included16 patients in chronic phase (CP)whom considered as optimal responder to IM (control group). Total RNA was extracted from PB and reverse transcription was done. Quantitative reverse transcription real time polymerase chain reaction (QRT-RT-PCR) was performed using specific primers to miRNA-150.Results showed that there was no significant difference in expression level of miRNA 150-1 between two groups of patients.

Keywords: Chronic myeloid leukemia, Imatinib, miRNA150-1, expression level, prognostic marker.

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INTRODUCTION

Mammalian microRNAs (miRNA) are short non-coding RNAs that regulate gene expression by inhibiting translation of specific target mRNAs[1].Many cellular functions regulated by microRNAs such as development altiming, apoptosis, cell proliferation, signal transduction and tumor genesis [2,3,4].Gene expression and epigenetic regulation by miRNAs were take attention in studying human malignancies and searching for regulators of miRNA expression levels which have clinical value in developing new treatment approaches inCML. Many miRNA sare located at chromosomal breakpoints and genomic regions associated with cancer [5,6,7.Expression of specific micro RNAs had been found in specific haemato logical cell types with a regulatory function in early haematopoietic differentiation, granulopoiesis, erythropoiesis, lymphoid development and megakaryopoiesis. An average of 2‑3 miRNAspresent as polycistronic clusters usually placed in hotspot regions prone to deletions and other molecular damaging alterations.This polycistron is regulated transcriptionally by c‑MYC. Also, oncogenic variants of ABL1 are up regulated by c‑MYC expression its required and acts synergistically with BCR‑ABL1, resulting in transformation to CML [8].It was referred to that hsa-miR‑150 among the most commonly deregulators miRNAs in CML [9]. A miRNA may function as a tumor suppressor or an on cogene. Cluster of miRNAs also exhibits increased expression in the early chronic phase of CML, but not in blast phase (BP), making it a possible therapeutic target [10].

Expression of miRNA in a group of mononuclear and CD34cells separated from bone marrow of CML patients at diagnosis was studied and it was found that miR-150, miR-221, miR-127, miR-16 were abnormally expressed and other miRNAs differentially expressed inmono nuclear cells including miR-150, miR-126, miR-221,miR-222, miR-21[11].

Regarding the correlation with treatment responsiveness, it was referred to that expression level change in specific miRNAs associated with resistance or responsiveness to imatinib after the treatment initiation inCML patients. The expression ofmiR-150 and miR-146ais relatively rapid enhancement and expression of miR-142-3p andmiR-199b-5pdecrease in peripheral blood mononuclear cells of newly diagnosed patients with CML two weeks after imatinib initiation
[12,13].

The current study aimed to characterize differentially expression level of miRNA-150 in peripheral blood of patients at different phases of CML including CP with major molecular response to IM and non-responder patients in AP and BC, including therapy failure to IM and NIL using Q-RT-RT-PCR to assessed the correlation betweenmiRNA-150 expression level with pathogenesis of CML and responsiveness to TKIs.

MATERIAL AND METHODS

Patients

In the current cross-section study,(34)PB samples were collected from CML patients recruited to the National Center of Hematology, Baghdad, Iraq, from April 2014 to April 2015. Determination of BCR-ABL1ratio was done using Q-RT-RT-PCR on authorized laboratory and patients were classified according to molecular response to: molecular non-responders whom have >0.1% BCR-ABL, Major Molecular Response(MMR) whom have 0.1% BCR-ABL, Complete Molecular response(CMR) whom have ≤0.01% BCR-ABL [14].

CML patients were grouped according to CML phase and molecular responsiveness to first line TKI treatment, imatinib (400mg/day) and second line TKI treatment, nilotinib, based on hematological and molecular findings(at sampling) into: Group-1 included (4) AP patients and(14)BC patients whom not showed molecular response to IM considered as non-responders and they switched to nilotinib(800 mg/day) due to primary, secondary failure or drug intolerance. Of them,(3/14) of BC patients and (1/4) of AP patient were non-responders to nilotinib [15].

Group-2 included (16)CP patients whom showed CMR considered as optimal responder to IM (400 mg/day) as control group. Peripheral blood sample(1ml)was collected from each patients in PAXgene® Blood RNA Tube IVD (cat no 762165 Qiagen, Hilden,Germany) and stored at-20 ºC.

The research was approved by the Ethical committee of the National Center of Hematology, Baghdad, Iraq.

miRNA extraction

The PAXGene® Blood miRNA kit (Cat no.763134,Qiagen) was used to extract total RNA fromPB samples following manufacturer instructions. Concentration and purity were determined for extracted products using Nano drop(Bioner, Korea).

Quantitative Reverse transcriptase Real Time polymerase chain reaction (Q-RT-RT‑PCR)

Total RNA were transcribed to cDNA using miscript II RT kit(Cat. No. 218160,218161) following manufacturer instructions. Buffer miScriptHiSpec(5X) was used to prepare cDNA. Quantitative Reverse transcriptase Real Time-PCR was performed on the Micthermal cycler (bio molecular system, USA) usingmiScript SYBR Green PCR kit (Cat. No. 218073,Qiagen,USA) to amplify the cDNA following manufacturer instructions .Primer hs-miR-150-1 miScript (Cat. No. MS00003577,Qiagen-USA) was used to quantified the expression level of miR-150-1 in extracted miRNA from studied subjects.Primerhs-SNORD61-11 (Cat. No. MS00033705,Qiagen,USA) was used to quantified the level of SNORD61 gene as housekeeping gene. A serial 2-fold dilutions of the cDNA were examined for each sample to determine the efficiency of reaction run. Then,2 µl from appropriate cDNA dilution was added to reaction mixture.

Post-amplification, a melting curve program was run at 95˚C for 1 min, 65˚C for 2 min followed by 65‑95˚C at 2˚C/min (data collection step).Relative fold changes of gene expression were assessed using $2^{-\Delta\Delta Ct}$ method [16]. Results were presented as expression fold change of a patients to control group.A cut‑off cycle threshold (Ct) value of 35˚C was used for miRNA-150-1 expression.

Statistical analysis

Data were summarized, analyzed and presented using statistical package for social science (SPSS version 23.0) and MedCalc version 15. Kolmogorov Smirnov test was used to identify variables with normal distribution. Normally distributed numeric variables were expressed as mean and standard deviation whereas those variables, not-normally distributed, were presented as median and inter-quartile range. Independent samples t-test was used to compare mean of normal variable between any two groups whereas, Mann Whitney U test was used to compare mean rank of non-normally distributed variables between any two groups. Categorical variables were expressed as number and percentage. Chi-square test with or without correction and Fischer exact tests were used to study association between categorical variables. The level of significance was chosen at P≤ 0.05.

RESULTS

Patient characteristics.

Expression profiles of miRNA 150-1 was analyzed in a total of 34CML patients on IM.The mean age of the included patientswas 39.32±10.95 years. The median duration of treatment by IMwas 6.03 ±2.49 years (range1-11 years). Male to female ratio was 1:1.25,table 1.

Table 1: Characteristics of the studied patients groups.

Characteristic	Group 1 n = 18	Group 2 n = 16	Total n = 34	P-value
Age (years)	38.00 ±12.31	40.81 ±9.34	39.32 ±10.95	0.463*
Imatinib Dose (mg/day)	433.33 ±76.70	400.00 ±0.00	417.65 ±57.58	0.092*
Duration of treatment (years)	6.39 ±2.17	5.63 ±2.83	6.03 ±2.49	0.381*
Gender (M/F)	8/10	10/6	18/16	0.292†

Data are expressed as mean standard deviation or number / number; n: number of cases; Group 1: CML with either Blast crisis or accelerated phase; group 2: CML with chromic phase "optimum responder to first line treatment" ; *: Independent samples t-test; †: Chi-square test

Expression level profile of miRNA-150-1

Regarding determination of BCR/ABL ratio in treated patients with IM using Q-RT–RT PCR to estimate molecular response level,it was found that the average of BCR/ABL ratio at samplingin PB samples of CML patients in-BC and APwassignificantly higher than that of CML patients in CP whom considered as optimum responder to IM($p<0.001$),table 2.

Regarding the miRNA 150-1 expression level, it was shown that the difference between the average of Ct of miRNA 150-1 in CML patients inBC and AP and that in CML-CP patients whom considered as optimum responder to IM was non-significant (P=0.679). The difference between the average of Ct of SNORD61 (housekeeping gene) gene expression level between all studied patientswas non-significant (P=0.945) and showed stable expression level across all samples analyzed (stable Ct/ngmiRNA), that referred to correct chose of this gene for normalization.

The difference between the average of ΔCt of miRNA 150-1 expression level in CML patients in BC and AP and that in patients in CP whom considered as optimum responder to IM was non-significant(P=0.558).

Table 2: Comparative results of BCR/ABL ratio and miRNA-150-1expresion level between two groups ofCML patients included in the study

Characteristic	Group 1 n = 18	Group 2 n = 16	P-value *
Q-PCR bcr-ablof (%)	3.600 (3.810)	0.002 (0.010)	<0.001
Average ofCtof miRNA 150	30.755 (5.950)	30.335 (5.170)	0.679
Average of Ct of miRNA 61	32.045 (3.160)	32.245 (3.080)	0.945
Average of ΔCt	-1.140 (2.200)	-1.035 (2.640)	0.558

Data are expressed as median (inter-quartile range); n: number of cases; Group 1: CML patients with either BC or AP phase whom non-responder to IM.Group 2: CML patients with CP phase whom optimum responder to IM. *: Mann Whitney U test.

The average of fold change of miRNA 150-1 expression level of CML patients in AP and BC patients was -1.97 ±2.08and that referred to there was down-regulation in gene expression about 2-fold from normal level of expression.

The scatter plot was done as shown in Fig.1 to study the correlation between BCR-ABL ratio and fold change in expression level of miRNA-150-1 to determine the direction and strength of the relationship between these variables.The Spearman Correlation Coefficient (Rho) value between BCR-ABL1 ratio and fold change of miRNA-150 expression level was 0.289 (P= 0.229). That referred to there was non-significant correlation between BCR-ABL ratio and fold change ofmiRNA-150 gene expression level.

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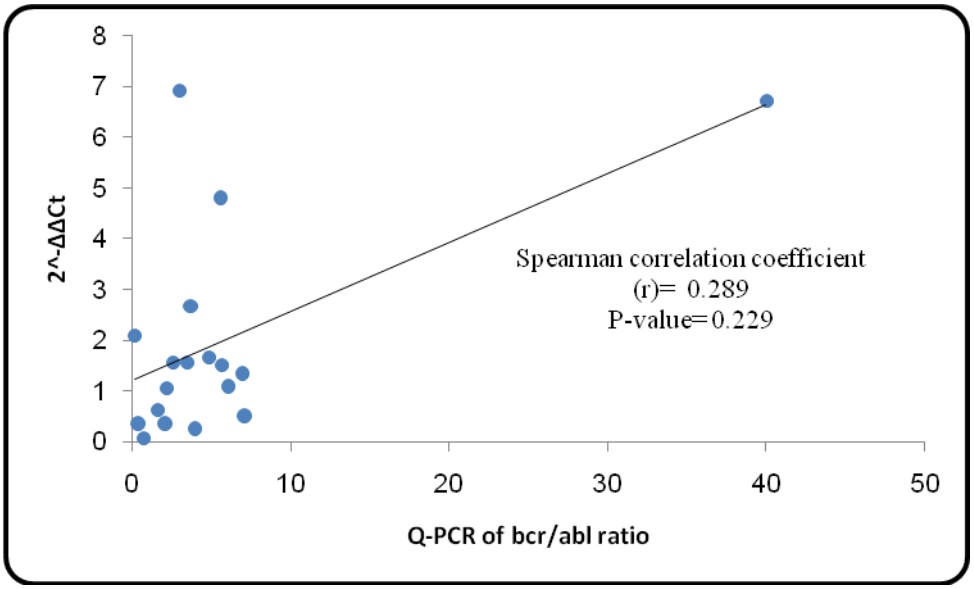

Figure 1: Spearman rank correlation between Q-PCR ration of BCR-ABL gene and 2^-ΔΔCt of miRNA 150-1.

Sensitivity and specificity of obtained results was calculated using Receiver Operator Characteristic (ROC) curve, Fig. 2 and table3.

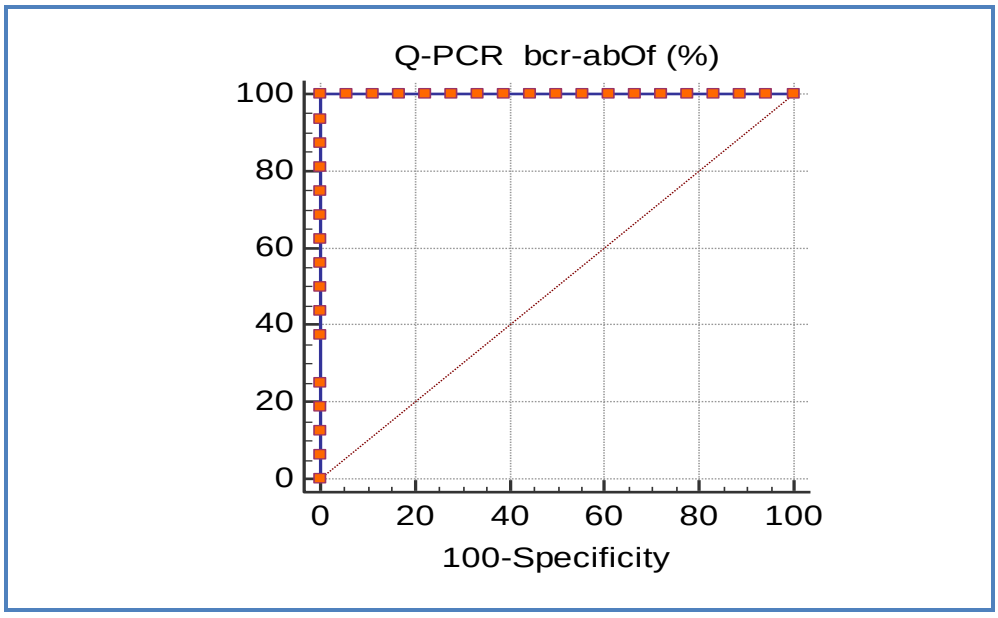

Figure2: Receiver Operator Characteristic (ROC) curve for abof % cut-off value calculation.

Table 3: ROC parameters of date

Abof % Cutoff value	≤0.06
AUC (95% CI)	1.000 (0.897-1.000)
P-value	<0.001
Sensitivity	100.00
Specificity	100.00

AUC: area under curve; CI: confidence interval

DISCUSSION

Early diagnosis of CML prior to blast crises phase has a significant value on patient survival rates. miR-150 has constantly been detected to be down-regulated in CML patientsthrough multiple studies making it a promising candidate marker for early CML diagnosis. Many studies referred to that lowered expression of miR-150 represents poor prognosis and a more advanced state of CML, while restoration of miR-150 was found to lessen symptoms in cell lines, suggesting that the down-regulation of this miRNA plays a role in disease initiation [11,12,17,18].

In the current study, data showed thatexpression level of miRNA-150-1usingQ-RT-RT-PCRconfirmed down-regulation in studied patients and there was similar expression level of miRNA-150 in CML patients in CP whom responders to IM with CMR and those in AP/BC whom failure to achieve molecular response.

Evidence for down-regulation of miR-150 as a diagnostic biomarker of CML was shown in a study used quantitative reverse transcription polymerase chain reaction approach on 50 samples from newly diagnosed CP-CML, and it was found significant down-regulation of miR-150[19].A studywas done using a microarray analysis on 10 samples from CP-CML patient alsoconformed the hypothetical of miR-150 down-regulated in CPas a biomarker for CML diagnosis[13]. A studyreferred to that while miR-150 may have potential for the diagnosis of CML, it cannot considered as biomarker to distinguish between CP and BC[20].

The profile of a grope of miRNAs expression levels were studied using microarrays and Q-RT PCR, and the results referred to that miR-150 was down-regulated in both CP and BP. Also,the results showed that miR-150 expression levels were not restored in patients developing resistance to Imatinib treatment[21]. It could be concluded that lower expression levels of miR-150 are indicative of poor prognosis for patients receiving TKIs treatment, and miR-150 expression level could be used as a biomarker for drug response.

The down-regulation of miR-150 was reported in the BCR-ABL transformed leukemia cell line Mo7e-p210 (megakaryoblast), and it was referred to the possibility of restoration in response to Imatinib treatment [11].

In the current study, it was found that the correlation between BCR-ABL ratio and fold change of miRNA-150 expression levelin both studied groups of patients was non-significant.Note that, 3/14 of CML patients in BC were also failure to nilotinib. That referred tomiRNA-150expression level is not efficient marker in predicting outcome and that agreed with the results of previous studies that referred to the similar findings [21,22]

CONCLUSION

Variations in results obtained in current pilot study and other previous studies may be caused from small sample sizes,different objectives, differences in the methodology used, making comparison difficult.
It is recommended for research on a large scale sample size to validate the possible role of miRNA expression as a prognostic marker. The use of other types of miRNA in relation with their signaling pathways regulators may be of value as prognostic markers in CML, which may lead to individualized therapy and improved patient outcome.

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