Circulating miR-146a expression predicts early treatment response to imatinib in adult chronic myeloid leukemia

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Accepted 20 October 2020
Published Online First 10 November 2020

ABSTRACT

This study aimed to investigate the prognostic role of circulating miR-146a in the prediction of early response to imatinib treatment in patients with chronic myeloid leukemia (CML). Sixty patients with CML and 20 healthy controls were recruited in this study. *BCR-ABL* was assessed by quantitative rt-PCR at days 0 and 90 of imatinib therapy. Circulating miR-146a levels were assessed by quantitative rt-PCR at days 0, 14 and 90 of imatinib therapy for patients and once for controls. At day 90 of treatment, treatment response was achieved in 48 patients (80.0%). Responders had significantly lower baseline Sokal score when compared with non-responders. They also had significantly lower *BCR-ABL* expression at day 90 of treatment. The circulating miR-146a level was significantly lower in patients with CML than in healthy subjects and showed a significant rise after 14 days of imatinib treatment and an inverse correlation with *BCR-ABL* expression levels at 90 days. Using multivariate logistic regression analysis, baseline *BCR-ABL (%)* (OR (95% CI) 1.09 (1.03 to 1.016), p=0.006) and miR-146a at 14 days (OR (95% CI) 0.002 (0.0 to 0.09), p=0.001) were significant predictors of treatment response. Using ROC curve analysis, it was found that miR-146a expression at 14 and 90 days could distinguish responders from non-responders (AUC (95% CI) 0.849 (0.733 to 0.928) and 0.867 (0.755 to 0.941), respectively). This study reported for the first time that measurement of the circulating miR-146a expression at 14 days can predict the early response to imatinib treatment in patients with CML. Thus, this work indicates that miR-146a should be investigated in the setting of treatment response to other tyrosine kinase inhibitors.

Significance of this study

What is already known about this subject?

- Chronic myeloid leukemia (CML) is characterized by *BCR-ABL1* translocation.
- Tyrosine kinase inhibitors (TKIs) as imatinib can inhibit *BCR-ABL1* and are used in CML treatment and thus, *BCR-ABL1* is used for monitoring the treatment response.
- MicroRNAs (miRs) are short non-coding RNAs that preferentially regulate gene expression.

What are the new findings?

- First study to assess the miR-146a as a predictor for early response to imatinib treatment in patients with CML.
- Circulating miR-146a level was significantly lower in patients with CML than in healthy subjects.
- Circulating miR-146a level showed a significant increase after 14 days of imatinib treatment specifically in the responders.
- An inverse correlation exists between miR-146a and BCR-ABL expression levels at 90 days.

How might these results change the focus of research or clinical practice?

- miR-146a at day 14 of imatinib treatment was suggested as a significant predictor of treatment response with high predictive value. These results should lead to further research on the role of miR-146 in the treatment response to other TKIs.

To cite: Habib EM, Nosiar NA, Eid MA, *et al*. *J Investig Med* 2021;**69**:333–337.

INTRODUCTION

Chronic myeloid leukemia (CML) is a clonal myeloproliferative disease characterized by t(9;22)(q34;q11) translocation generating an oncogenic fusion gene known as *BCR-ABL1*.[1] The untreated CML can progress from an initial indolent chronic phase into an accelerated phase and/or a blast phase.[2] Tyrosine kinase inhibitors (TKIs), most notably imatinib, has been used in the treatment of CML. They act mainly by inhibiting *BCR-ABL*. Although imatinib has emerged as the most effective therapeutic regimen in CML, imatinib resistance was detected in a considerable number of CML cases.[3]

Mammalian microRNAs (miRs) are a large class of small non–protein-coding RNAs which can bind specifically to the 3′-untranslated regions (3′-UTR) of target mRNAs either complementarily or partially complementarily resulting in transcriptional and or translational repression.[4] miRs promote and modulate

various cellular functions such as signal transduction and apoptosis as well as cellular development, proliferation, differentiation, and migration.[5 6] Thus, miRs play a pivotal role in tumorigenesis and currently, their role is extensively reported in various human malignancies.[7–11]

In CML, several miRs have been reported to stimulate or repress tumor growth. For example, miR-199-5b may enhance tumor progression in CML via inhibition of Hes 1, a transcriptional factor in the Notch pathway.[12] Moreover, miR-138 was reported to bind to the coding region of *BCR-ABL* and 3′-UTR region of cyclin D3 (CCND3) inhibiting their transcription.[13] Furthermore, BCR-ABL was reported to promote the CML progression by activating the nuclear-factor-κB (NF-κB) pathway. It was found that overexpression of miR-146a suppresses several factors of the NF-κB pathway resulting in apoptosis.[14] Thus, the miR-146a overexpression in CML on imatinib treatment was suggested to increase the sensitivity of CML cells to apoptotic signaling.[15] The aim of this study is to assess the circulating miR-146a levels and its role as a predictor for early hematological and molecular response to imatinib treatment in patients with CML.

SUBJECTS AND METHODS

The present study was conducted at Kafr El-Shiekh and Tanta Universities Hospitals, Egypt during the period from September 2017 to June 2019. The study protocol was conducted according to the Helsinki declaration and approved by the Institutional Review Boards of both Universities and informed consent was obtained from all participants. The cross-sectional study included 60 patients with newly diagnosed CML in chronic phase. Patients were diagnosed according to revised 2016 WHO criteria.[16] Patients were excluded if they had other malignant diseases or if they had abnormal liver or kidney functions. In addition, there were 20 age-matched and sex-matched healthy controls. All patients had careful history-taking, thorough clinical examination, and routine laboratory investigations (complete blood count, liver and renal function tests). All patients received the same standard therapy which is imatinib (first-generation TKI) in a dose 400 mg/day for 3 months.[17] *BCR-ABL* was assessed by quantitative rt-PCR at days 0 and 90 of imatinib therapy. Patients were classified into those with low, intermediate, and high risks according to Sokal score. Sokal score is a prognostic score for patients with CML that comprises patients' age, splenic size, platelet number, and blast cell percentage.[18] Circulating miR-146a levels were assessed by quantitative rt-PCR at days 0, 14, and 90 of imatinib therapy for patients and once for controls. The results were interpreted separately by independent investigators who were blinded to other laboratory results and clinical findings of the patients. The primary endpoint of the present study is early treatment response (ETR) at 90 days. ETR is defined by complete hematological response (defined by white blood cell count less than 10×10^9/L with no immature granulocytes, basophils less than 5%, platelet count less than 450×10^9/L, and non-palpable spleen) and early molecular response (identified as *BCR-ABL1* transcript less than 10% for 3 months).[17]

Relative quantitation of miR-146a expression by rt-PCR

Sample preparation

Total RNA, including miRs, was isolated from EDTA plasma samples using miRNeasy Mini Kit (QIAGEN, Maryland, USA, Cat No. 217004) according to the manufacturer's directions. The RNA yields were measured at 260, 280, and 230 nm using NanoDrop2000 Spectrophotometer (Thermo Scientific, USA) to assess their concentration and purity.

Reverse transcription

In the reverse transcription (RT) step, the RNA yields were used as a template for cDNA synthesis using TaqMan MicroRNA Reverse Transcription Kit and a small RNA-specific, stem-loop RT primer from the TaqMan Small RNA Assays.

RQ-PCR

The cDNA amplification was performed on Stratagene Mx3000p real-time instrument using TaqMan Small RNA Assay together with TaqMan Universal PCR Master Mix II. RNU48 was used as an endogenous control. The primers sequences used were as follows: for miR-146a (forward: 5′-ACTGAATTCCATGGGTTGTGTC-3′, reverse: 5′-TGACAGAGATATCCCAGCTGAAG-3′), for RNU48 (forward: 5′-AGTGATGATGACCCCAGGTAA-3′, reverse: 5′-GTGATGGCATCAGCGACACA-3′). The fluorescence signals were collected at the end of each cycle and displayed in relation to the cycle numbers. The baseline and threshold values were automatically adjusted by the software and the cycle threshold (C_t) for each sample was calculated using the software.

Data analysis and result interpretation

The circulating miR-146a expression was relatively quantified using the comparative C_t method. The C_t of miR-146a was normalized for the endogenous control C_t (RNU48) and compared with C_t of the normal control using an arithmetic formula ($2^{-\Delta\Delta Ct}$) to achieve relative quantification.[19]

Statistical analysis

Data were processed using IBM SPSS software package V.20.0. Graphs were designed on GraphPad Prism V.8 software. Categorical data were presented as number and per cent and compared using χ^2 test. Numerical data were described as mean and SD or median and range and compared using Student's t-test or Mann-Whitney U test. Pearson correlation analysis was performed between *BCR-ABL* and miR-146a expressions at day 90 of imatinib treatment. Logistic regression analysis was used to identify predictors of treatment response. Receiver operating characteristic (ROC) curve analysis was used to identify the ability of miR-146a at 14 and 90 days to distinguish responders from non-responders. Youden's index was used to determine the optimal cut-off value of miR-146a. A statistically significant p value was less than 0.05.

RESULTS

The present study included 60 patients with newly diagnosed CML and 20 age-matched and sex-matched healthy controls. Baseline characteristics of the studied groups are shown in table 1.

At baseline, a comparison between patients and controls revealed significant higher miR-146a in controls as compared with patients ($p<0.001$) (figure 1A). At 14 and 90 days of imatinib treatment, there was a significant

Table 1 Comparison between patients and controls regarding the baseline data

	Patients n=60	Controls n=20	P value
Age (years)	55.15±10.95	51.2±9.25	0.365
Female/male (n)	27/33	10/10	0.685
WBC (×10^9/L)	91.23±39.5	9.20±1.98	<0.001*
Hb (g/dL)	10.95±1.36	13.1±1.62	0.031*
Platelets (×10^9/L)	430.7±88.72	360.6±79.1	0.018*
ALT (U/L)	45.3±8.2	40.6±7.98	0.321
AST (U/L)	46.8±12.6	44.1±10.3	0.126
Creatinine (mg/dL)	0.91±0.21	0.79±0.19	0.081
BUN (mg/dL)	13.8±4.25	12.8±4.01	0.401

*P<0.05 significant.
ALT, alanine transaminase; AST, aspartate transaminase; BUN, blood urea nitrogen; n, number; WBC, white blood cell.

increase in miR-146a levels in the patient group compared with the baseline levels (p<0.001) (table 2, figure 1B). At day 90 of treatment, there was a significant decline in BCR-ABL expression levels (p<0.001) which showed an inverse correlation with miR-146a levels (r=−0.288, p=0.025) (figure 1C).

Treatment response was achieved in 48 patients (80.0%). Comparison between responders (n=48) and non-responders (n=12) regarding baseline Sokal score and molecular markers is shown in table 2. Responders had significantly lower baseline Sokal score when compared with non-responders (1.04±0.18 vs 1.28±0.23, p=0.031). While there were no significant differences between responders and non-responders regarding baseline *BCR-ABL* expression, responders had significantly lower *BCR-ABL* expression at day 90 of treatment (5.56%±2.16% vs 18.00±6.22, p<0.001). Moreover, responders showed

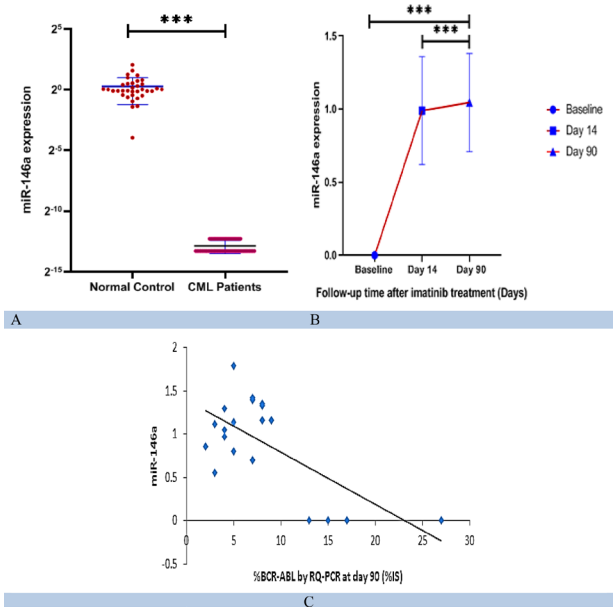

Figure 1 miR-246a expression (A) in patients with chronic myeloid leukemia (CML) vs normal control, (B) during the follow-up period of imatinib treatment, (C) correlation with *BCR-ABL* expression at 90 days after imatinib treatment. ***p<0.001.

significantly higher miR-146a than the non-responder at day 14 of imatinib treatment (1.016 vs 0.619, p<0.001) and at 90 days (1.153 vs 0.749, p<0.001).

Using multivariate logistic regression analysis, significant predictors of treatment response were baseline *BCR-ABL* (%) (OR (95% CI) 1.09 (1.03 to 1.016), p=0.006) and miR-146a at 14 days (OR (95% CI) 0.002 (0.0 to 0.09), p=0.001) (table 3).

Using ROC curve analysis, it was found that miR-146a expression at 14 and 90 days could distinguish responders from non-responders (AUC (95% CI) 0.849 (0.733 to 0.928) and 0.867 (0.755 to 0.941), respectively). The optimal cut-off of miR-146a expression was >0.757 (68.7% sensitivity and 83.3% specificity) and >0.852 (75.0% sensitivity and 83.3% specificity) at 14 days and 90 days of imatinib treatment (figure 2).

DISCUSSION

The main pathogenic mechanism underlying the progression of CML is the presence of *BCR-ABL1* fusion gene which promotes the tyrosine kinase activity with subsequent activation of different downstream signaling cascades. Therefore, the main therapeutic target in CML is the inhibition of tyrosine kinase activity. Imatinib mesylate (Gleevec or Glivec—formerly STI571), an ABL kinase inhibitor, which has revolutionized the CML therapy, is a selective TKI, which contests with ATP in binding the BCR-ABL (and ABL) protein kinase.[20] It produces sustained complete hematologic responses in patients with CML.[21] According to the recent treatment and follow-up guidelines, it is crucial to follow up and monitor the treatment response after the start of TKI administration in order to early detect any therapeutic resistance. Hence, a more appropriate intervention can be applied in a suitable time such as increasing the therapeutic dose or changing to another type of TKIs. Thus, molecular monitoring of *PCR-ABL1* by reverse transcriptase quantitative PCR (RT-qPCR) is done at regular intervals.[22]

In the present study, treatment response was achieved in 80% of patients with CML. Responders had significantly lower Sokal score when compared with non-responders in agreement with previous reports. This results agreed with those reported that the Sokal score can be used effectively as a predictor for the complete cytogenetic response and/or complete molecular response in patients with CML after imatinib treatment.[23–25] However, the predictive efficacy of Sokal score had not been detected in other studies.[26 27] In addition, we found that responders had significantly lower *BCR-ABL* expression at 90 days when compared with non-responders. This is attributed to the fact that imatinib, dasatinib, and nilotinib, the first-line TKIs, can inhibit the altered kinase activity in CML induced by *PCR-ABL* oncogene.[17] Also, assessment of *PCR-ABL* transcripts by standardized molecular techniques regularly throughout the TKI treatment period is crucial to improve the outcome.[28]

The main therapeutic target of TKIs in patients with CML is to suppress its progression from chronic phase to the accelerated or blast phases which are usually unresponsive to treatment and also to promote the disease remission and appropriate outcomes. Thus, searching for novel markers for early prediction of treatment response in CML is emerging.[29] Evidence suggested that miRs play a pivotal

Table 2 Comparison between responders and non-responders regarding Sokal score and molecular marker expression

	All patients n=60	Responders n=48	Non-responders n=12	P value
Baseline Sokal score mean±SD	1.085±0.211	1.04±0.18	1.28±0.23	0.031*
Baseline *BCR-ABL* (%) mean±SD	37.15±13.64	36.0±14.27	41.75±11.21	0.466
BCR-ABL at 90 days (%) mean±SD	8.05±5.99	5.56±2.16	18.00±6.22	<0.001*
Baseline miR-146a median (IQR)	0.0001 (0.0001–0.0002)	0.0001 (0.0001–0.00018)	0.0002 (0.0001–0.0002)	0.001*
miR-146a at 14 days median (IQR)	0.888 (0.619–1.4)	1.061 (0.712–1.454)	0.619 (0.554–0.742)	<0.001*
miR-146a at 90 days median (IQR)	1.08 (0.8–1.32)	1.153 (0.883–1.343)	0.749 (0.639–0.853)	<0.001*

*P<0.05 significant.

role in maintaining various cellular functions that are related to cell growth and homeostasis, and thus promoting CML progression and TKI resistance. Therefore, miRs were suggested as potential molecular biomarkers for detecting and monitoring the CML progression as well as predicting the treatment resistance.[30]

In this study, we aimed to investigate the potential role of circulating miR-146a expression as a predictor for the early hematological and molecular response after imatinib treatment. We found that circulating levels of miR-146a was significantly lower in patients with CML than in healthy subjects and showed a significant rise after 14 days of imatinib treatment in patients with CML specifically in the responders when compared with the non-responders. Moreover, our study showed an inverse correlation between *BCR-ABL* expression levels and miR-146a levels at 90 days. Therefore, the present study showed for the first time that 14-day levels of miR-146a could efficiently predict early response to imatinib treatment with an acceptable efficacy (AUC=0.849).

Evidence suggested that there is a marked variation in the expression patterns of circulating miRs between the untreated chronic phase patients with CML and those treated with imatinib. Thus, miRs can be used as predictive markers for monitoring treatment response and prognosis of patients with CML.[20] O'Connell *et al*[31] stated that the altered expression of miR-125, miR-146, miR-155, and miR-223 can promote the progression of myeloid malignancies based on their functional regulatory role in myelopoiesis. Ferreira *et al*[20] reported that imatinib could alter miR-15a and miR-130a levels and the miR-26a, miR-29c, miR-130b, and miR-146a expressions were decreased in imatinib-resistant patients compared with imatinib-responsive patients. However, they did not assess the miR expression relative to the treatment response in a time-dependent manner. Two weeks following TKI treatment in 10 patients, Flamant *et al*[12] reported an altered expression pattern of miRs in patients with CML with miR-150 and miR-146a upregulated, and miR-142-3p and miR-199b-5p downregulated, which endorse the capability of TKIs in regulating the expression pattern of miRs within tumor cells. Interestingly, they observed that, in only one patient, the expression pattern was different as miR-146a expression level was found to be decreased by day 14 of treatment relative to the baseline level. Although this patient developed complete hematological remission, he, unfortunately, developed acute hepatitis which forced him to stop the imatinib treatment after 4 months and this may explain the varied miR-146a expression in this patient.[12] However, this study only assessed the change in miR-146a expression on imatinib treatment without relating it to the treatment response.

In CML, *BCR-ABL* has been reported to promote the transcriptional activity of several members of NF-κB machinery resulting in increased cell growth and proliferation. Thus, suppression of the activity of NF-κB in cells expressing *BCR-ABL* results in apoptosis.[14] miR-146a was found to suppress the expression and transcriptional activity of *IRAK1* and *TRAF6* with subsequent deregulation of NF-κB signaling cascade.[30] Therefore, downregulation of miR-146a mediates the oncogenic and anti-apoptotic activity of *BCR-ABL* via synergistic activation of NF-κB signaling.[32]

Although the findings of the present study are limited by the small number of included patients as well as the relatively short duration of treatment monitoring, our results can provide a guide for upcoming studies. Therefore, we recommend further validation of our results in future studies with larger cohorts. In conclusion, this study shows

Table 3 Predictors of treatment response at 90 days

	Univariate analysis		Multivariate analysis	
	OR (95% CI)	P value	OR (95% CI)	P value
Age	0.98 (0.96 to 0.99)	<0.001*	–	–
Sex	0.5 (0.23 to 1.11)	0.09	–	–
Sokal score	0.35 (0.2 to 0.6)	<0.001*	–	–
Baseline *BCR-ABL*	0.97 (0.95 to 0.99)	<0.001*	1.09 (1.03 to 1.016)	0.006*
miR-146a expression at 14 days	0.16 (0.07 to 0.36)	<0.001*	0.002 (0.0 to 0.09)	0.001*

*P<0.05 significant.

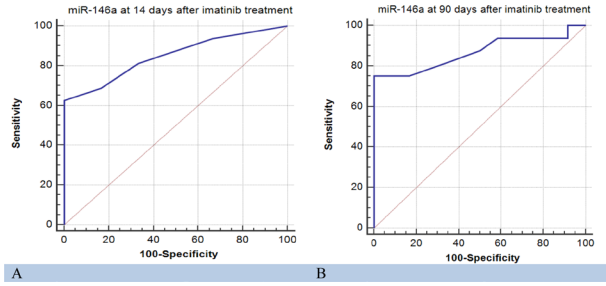

Figure 2 Receiver operating characteristic curve for miR-146a expression and treatment response (A) after 14 days, (B) 90 days after imatinib treatment.

that circulating levels of miR-146a can predict the early treatment response in patients with CML after 14 days of imatinib treatment. These findings suggested miR-146a as a potential molecular marker for monitoring the response to TK intervention and prediction of early therapeutic resistance which can be properly managed by adjusting TKI doses or changing to another TKI line.

Contributors EMH, MAE, MTAG, DES, AEH, NAN: study designing, retrieving the literature, performing routine and molecular investigations, analyzing data. AMT: patients' selection and follow-up. All authors: writing the original draft, reviewing and final manuscript approval.

Funding The authors have not declared a specific grant for this research from any funding agency in the public, commercial or not-for-profit sectors.

Competing interests None declared.

Patient consent for publication Obtained.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement All data relevant to the study are included in the article.

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