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Clinical Significance of Microrna-21 and Its Role as A Predictor of Therapeutic Response to Tyrosine Kinase Inhibitors in Egyptian Chronic Myeloid Leukemia Patients

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Abstract:

Background: Chronic myeloid leukemia (CML) is a myeloproliferative neoplasm with a prevalence of one to two patients per 100,000 adults. This investigation aimed to assess the clinical significance of microRNA-21 in CML & its role as a predictor of therapeutic response to tyrosine kinase inhibitors (TKI) in an Egyptian patient. **Patients and methods**: This case-control research has been performed on ninety cases; forty-five of them had chronic myeloid leukemia, fifteen of them had recently been diagnosed with chronic myeloid leukemia, fifteen patients on Imatinib, fifteen patients on second-generation TKI after failure of Imatinib, & 45 healthy controls. Patients were selected from internal medicine & hematology clinics in Beni-Suef University Hospital and Kasr-Alini Hospital in the period between April 2022 and April 2023. **Results**: MicroRNA-21 had a statistically significant role in CML disease prediction. At a cutoff of 1.2 with 53% sensitivity& 88% specificity. Also, microRNA-21 had a statistically significant role in the therapeutic response to tyrosine kinase inhibitors. At a cutoff of 0.73 with 86% sensitivity& 66% specificity. **Conclusion**: In CML cases, miRNA-21 expression levels were significantly greater than those in controls. (1.68±1.26, 0.90±0.22) (p-value, 0.0001). The levels of miRNA-21 expression in CML patients decreased after ttt with TKI being higher in TKI resistance patients. there was no correlation between micro-RNA 21 expression &BCR-ABL after 6 months of Imatinib (p-value 0.465). No correlation has been observed between the expression of micro-RNA 21 & the Sokal score (p-value 0.423).

Keywords: CML, microRNA-21, TKI

1. Introduction

A myeloproliferative neoplasm, CML has a prevalence of one–two cases per 100,000 adults. It is responsible for about fifteen percent of newly diagnosed patients with leukemia as adults. [1].

Small-molecule tyrosine kinase inhibitors (TKIS) have been developed that effectively interfere with the interaction between the adenosine triphosphate & BCR-ABL1 oncoprotein. Consequently, the malignant clone's cellular proliferation is blocked, dramatically changing the therapeutic landscape for chronic myeloid leukemia. This targeted strategy changes the natural history of chronic myeloid leukemia, resulting in a ten-year survival rate improvement from approximately twenty percent to ninety percent. [2,3]

Resistance to tyrosine kinase inhibitors can also develop independently of BCR-ABL, making new generations of small-molecule tyrosine kinase inhibitors ineffective. Around twenty percent of chronic myeloid leukemia cases develop either primary (i.e., lack of responsiveness) or secondary (i.e., loss of response after initial treatment) tyrosine kinase inhibitor resistance. [4]

MicroRNAs (miRs) are a group of small single-stranded non-coding RNAs (approximately twenty-two nucleotides in length) that play the role of epigenetic regulators of particular targets by regulating the expression of genes through mRNA cleavage or translational suppression. [5].

The unique molecular characteristic of chronic myeloid leukemia is the presence of BCR-ABL1, which is the target of management with TKI. The ability to resist imatinib & other small molecules of a tyrosine kinase inhibitor has been recognized as a common challenge in treating chronic myeloid leukemia. In addition to BCR-ABL1, it is possible that microRNAs are involved in the acquisition of medication resistance, as they are potent regulators that could regulate gene expression that is associated with medication transport or the activation of essential signaling pathways. The most deregulated microRNAs investigated in CML are microRNAs-150, microRNAs-130 b, microRNAs-204, & microRNAs-10a. However, several other microRNAs are emerging in the complex network of microRNAs. [6].

The development of neoplasia has been correlated to the overexpression of oncomiR miR-21, which targets numerous tumor suppressor genes that are involved in proliferation, survival, & apoptosis. Phosphatase & tensin homolog (PTEN), a negative modulator of the PI3K/AKT pathway, are frequently referred to as downregulated in cancer. This is an example of a miR-21 target. Furthermore, the acquisition of drug resistance, including gemcitabine & a small molecule of a tyrosine kinase inhibitor, has been associated with miR-21 levels. Phosphatase &tensin homolog are also targets of microRNAs (26b); however, the role of this microRNA in cancer remains controversial, being sometimes described as oncomiR & other times as tumor suppressor microRNA. [7].

The aim of this estimation was to assess the clinical significance of microRNA-21 in chronic myeloid leukemia & its role as a predictor of therapeutic response to tyrosine kinase inhibitors in Egyptian cases.

2. Patients and methods

This case-control investigation has been performed on ninety cases: forty-five cases with CML, 15 cases with newly diagnosed CML, 15 patients on Imatinib, 15 cases on 2nd generation TKI

after failure of Imatinib, & 45 healthy volunteers. Patients were selected from the internal medicine & hematology clinics at Beni-Suef University Hospital & Kasr-Alini Hospital between April 2022 & April 2023. CML was diagnosed based on the last NCCN guidelines, which included CBC with differential, BCR-ABL by RT PCR, & bone marrow aspirate & biopsy.

Exclusion criteria included BCR-ABL-ve CML patients & CML in Accelerated& Blast Phase.

Ethical considerations

This investigation has been conducted in accordance with the Helsinki Declaration. Following the Ethical Committee of Beni-Suef University Hospitals' approval, written informed consent has been obtained in all cases.

Methods:

All the cases have been exposed to the following: BCR-ABL1 by international standards in chronic myeloid leukosis cases at diagnosis & 3 months after tyrosine kinase inhibitor (TKI) treatment, history taking, clinical examination, laboratory investigations, & miRNA-21 evaluation by quantitative real-time polymerase chain reaction (RT-PCR).

miRNA-21 RNA extraction: Following the manufacturer's protocol, the miRNeasy Mini Kit (Qiagen Incorporated, 28159 Avenue, Stanford Valencia, CA 91355, the states) has been utilized to isolate the total RNA, which included small RNA, from plasma.

RNA purity and concentration measurements: three microliters of RNA solution have been added to seventy-two liters of di-ethylpyrocarbonate (DEPC) water (dilution 1: 25), followed by a vortex. The Nanodrop spectrophotometer, which was provided by Thermo Fisher Scientific at 3411 Silverside Road in Wilmington, the states, has been utilized to read the sample at 260 nanometers &280 nanometers. Protein has been detected at 230 nanometers. Samples have been considered to have good RNA purity if the A260/A280 ratio is between 1.8 and 2.

Reverse transcription: Utilizing the Taqman MicroRNA Reverse Transcription Kit in accordance with the manufacturer's protocol, reverse transcription (RT) has been conducted on the extracted RNA prepared during the preceding phase. MicroRNA-21-specific stem-loop primers & small nuclear miRNA-16 have been utilized as an endogenous control, all of which have been supplied by Applied Biosystems (Applied Biosystems, 850 Lincoln Centre Dr. Foster City, California, 94404, the state).

PCR amplification: The Taqman miRNA assay kits given by Applied Biosystems (850 Lincoln Centre, Foster City, California, 94404, state) have been utilized to conduct amplification on an RT-PCR system in accordance with the Minimum Information for Publication of Quantitative RT-PCR Experiments (MIQE) guidelines.
The miRNA Easy Plasma Kit integrates silica-membrane-based purification of total RNA with phenol & guanidine-based sample dissociation. The Qiazol Lysis Reagent, a monophasic solution of guanidine, thiocyanate, &phenol, is packaged with the kit. Its purpose is to simplify the process of lysis, denature protein complexes and RNAs, and eliminate the majority of the residual DNA & proteins from the lysate through organic extraction. Application of the miScript PCR system for miRNA quantification. RNA extraction & quantification. The Qiagen miRNA Easy reagent has been utilized to extract microRNA-21 from the plasma samples.

Total RNA purification, which includes small RNAs, from the sample:

The sample has been disrupted & homogenized utilizing an appropriate method after 1000 microliters of Qiazol Lysis Reagent have been added to 200 milliliters of the sample. The homogenate has been incubated at room temperature (fifteen to twenty-five Celsius) for five minutes. Then, 200 milliliters of chloroform have been added to the tube & capped securely. Shake vigorously for fifteen seconds. Subsequently, we incubated the samples at room temperature for two to three minutes. Subsequently, it was centrifuged at 12,000× g for fifteen minutes at four degrees Celsius. The upper aqueous phase has been transferred to a new collection tube. Avoid transferring any interphases. We added 1.5 volumes of hundred percent ethanol & mixed them thoroughly, utilizing a pipette. Pipetting up to 700 microliters of sample into a miRNeasy Mini column in a two-milliliter collection tube. The lid was closed, & centrifugation was conducted at room temperature for fifteen seconds at a rate of ≥8000 x g. We then discarded the flow-through. Step 7 is repeated, utilizing the reminder of the sample. We added 700 microliters of Buffer RWT to the RNeasy Mini spin column, gently closed the lid, & centrifuged at a rate of ≥8000 x g for fifteen seconds. The flow-through has been discarded. We pipetted 500 milliliters of buffer RPE into the RNeasy Mini spin column. Gently close the lid & centrifuge at a rate of ≥8000 x g for fifteen seconds. The flow-through has been discarded. After that, we added another 500 milliliters of buffer RPE to the RNeasy Mini spin column. Gently close the lid & centrifuge at a rate of ≥8000 x g for two minutes. The old collection tube with the flow-through has been discarded, & the RNeasy Mini spin column has been transferred to a new two-milliliter collection tube (not supplied). Then, centrifugation was conducted at full speed for one minute. The RNeasy Mini spin column has been transferred to a new 1.5-milliliter collection tube. In the final step, we directly pipetted 30–50 microliters of RNA-free water onto the RNeasy Mini spin column membrane. To elute the RNA, gently close the lid & centrifuge for one minute at a speed of ≥8000 x g.

Statistical Analysis

Data analysis has been conducted utilizing the Statistical Package of Social Science (SPSS) software version eighteen on Windows seven. Data manipulation has been facilitated by the collection & coding of the data, & it has been double-entered into Microsoft Access. Simple descriptive analysis in the form of percentages & numbers for qualitative data arithmetic means are used to quantify central tendency, while standard deviations are used to quantify dispersion for quantitative parametric. The quantitative data involved in the investigation has been initially checked for normality utilizing the one-sample Kolmogorov-Smirnov test in each group of the study. Subsequently, inferential statistical tests have been chosen. For quantitative data, an independent student t-test has been utilized to compare the measures of 2 independent groups of quantitative data. A one-way ANOVA test has been utilized to compare quantitative data from more than two independent groups, with Bonferroni post-hoc tests performed to estimate the significance of each group. Applying the Chi square test to qualitative data allows for the comparison of 2 or more qualitative categories. The P-value less than or equal to 0.05 has been regarded as the cut-off value for significance.

3. Results

Table 1 demonstrated that the mean age among controls was higher (40.91± 11.28) without significant difference. The cases were: 48.9% of them were men & 51.1% were women. There was statistically insignificant variance among the cases& the control groups according to sex (p-value higher than 0.05), while BMI showed significant variance (p-value 0.0001).

Table (1): General characteristics of the group of the study (Number=ninety)

		No (%)		
		Patients No= 45	**Healthy Controls No= 45**	**P-value***
Age (mean ±SD)		40.86±11.26	40.91±11.28	0.985
Sex	**Male**	22 (48.9)	20 (43.2)	0.589
	Female	23 (51.1)	25 (56.8)	
BMI	**Normal weight (20-24.9)**	14 (33.3)	27 (60)	0.001*
	Overweigh (25-29.9)	**20 (44.4)**	17 (37.8)	
	Obese (more than 30)	11 (24.4)	0	

*p-value higher than0.05 is considered insignificant by Chi-Square test for sex & t test for age & BMI. *BMI body mass index.

Table 2 demonstrates that there was a significant change in hemoglobin, WBCs, & platelets from baseline until 6 months of follow-up in TKI response & resistance patients. (P-value less than 0.05).

Table (2): Hematological Response (hemoglobin, WBCs & platelet) in Different CML categories (No=30):

Hemoglobin	**TKI response N0= 15**	**TKI resistance No= 15**	**P-value between groups**
Baseline (at time of diagnosis)	10.52±1.51	9.78±1.44	0.185
After 6 months	11.96±1.44	13.11±1.36	0.036*
P-value (pre-post)	0.0001*		
WBCs			
Baseline (at time of diagnosis)	157.06±81.37	198.93±107.46	0.239
After 6 months	9.00±6.93	10.0±9.96	0.754
P-value (pre-post)	0.0001*		
Platelets			
Baseline (at time of diagnosis)	570.86±453.02	576.00±649.84	0.980
After 6 months	323.73±187.04	211.73±59.81	0.036*
P-value (pre-post)	0.004*		

Table 3 demonstrates that there was a significant variance regarding microRNA-21 expression between studied CML patients & studied healthy controls, as mean values were higher in patients with a significant variance as the p value was lower than 0.05.

Table (3): MicroRNA-21 Expression of the study groups (No=90):

microrna-21 Expression	**Patients No= 45**	**Healthy Controls No= 45**	
(mean ±SD)	1.68±1.26	0.90±0.22	0.0001*
Minimum -maximum	0.09-6.15	0.10-1.17	

Table 4 demonstrates the mean values of BCR-ABL after 6 months among TKI responders &non-responders, with a significant variance as the p-value was 0.002.

Table (4): Comparison between TKI responder & non-responder regarding their BCR-ABL after 6 months (No=30):

BCR-ABL after 6 months	**TKI response No= 15**	**resistance TKI No= 15**	**p-value**
(mean±SD)	1.34±0.46	*10.62±11.27**	0.002*
minimum	0.02	5.30	
maximum	0.1	36.00	

*p-value less than0.05 is considered significant by one way a nova test..BCR Abl;breakpoint cluster region for ableson gene

Table 5 demonstrated that the mean values of the malignancy sokal score among patients' subcategories had a non-significant difference, as the p-value was 0.614.

Table (5): Comparison between patients regarding their malignancy sokal score (No=30):

Sokal score	**TKI response number = fifteen**	**Resistance TKI number= fifteen**	**p-value**
(mean±SD)	0.96±0.41	*1.02±0.20**	0.614
Minimum	0.60	0.70	
Maximum	1.90	1.40	

Table 6 showed that microRNA-21 had a statistically significant role in the prediction of CML disease. At a cutoff of 1.2 with 53% sensitivity & 88% specificity. Also, microRNA-21 had a statistically significant role in the therapeutic response to tyrosine kinase inhibitors. At a cutoff of 0.73 with 86% sensitivity & 66% specificity.

As demonstrated in **Table 7,** there was no correlation between micro-RNA-21 expression & BCR-ABL after 6 months (p-value 0.465).

Table (6): Cut off, sensitivity, specificity of in microrna-21 prediction of CML patients &heathy controls & new diagnosed & after TTT

microRNA-21	Area under curve	P-value	cut off		Sensitivity	specificity
CML patients	0.626	0.04	1.2		53%	88%
After TTT	0.756	0.017	0.73		86%	66%

Table (7): Correlation between Micro- RNA 21 & BCR-ABL after 6 months.

		Micro-RNA21
BCR-ABL after 6 months	Pearson Correlation	-0.141
	Sig. (2-tailed)	0.465

Discussion

The mean age among controls was higher (40.91±11.28) without significant difference. The cases were: 48.9% of them were men, & 51.1% were women. There was statistically insignificant variance among the patients & the control groups according to sex (p-value higher than 0.05), while BMI showed a significant difference (p-value 0.0001).

This goes in association with **Abbas & Al-Rubaie [8],** who analyzed sixty chronic myeloid leukemia cases divided into 2 categories based on how they responded to their management, whether they were resistant or sensitive to IM. The control group consisted of ten healthy, normal participants.

Our study revealed a hematological response after 6 months to imatinib treatment, showing a good hematological response with a statistically significant difference regarding hemoglobin, leukocytes, & platelet counts from baseline with a p-value less than 0.05 among both TKI responder & non-responder groups as hemoglobin increased & platelets &leukocytes reached the normal range.

This is also in agreement with **Suchiita et al. [9],** who showed the same hematological parameters after six months of treatment with imatinib.

In this study, we found that the mean values of the malignancy Sokal score among patients' subcategories were non-significant, as the p-value was 0.614. But the Sokal score mean value was lower in TKI responders (0.96±0.41) than non-responders (*1.02±0.20).*

This may go in agreement with **Yilmaz et al.'s [10]** findings, where the Sokal risk score has been estimated for all cases & no significant variance has been detected between the three groups (p = 0.493).

On the contrary, **Habib et al**. **[11],** who examined 60 patients with chronic myeloid leukemia & 20 controls who were sex- & age-matched, found that cases with chronic myeloid leukemia have been categorized regarding ETR at day ninety based on BCR-ABL1 transcript level results into group 1A cases with chronic myeloid leukemia with ETR (number = forty-eight) & group 1B patients with chronic myeloid leukemia with no ETR (number = twelve). Both groups exhibited a significant variance in the Sokal score & BCR-ABL1 transcript level at day ninety (P-value less than.001). In univariate analysis, cases' age & Sokal score were significantly associated with ETR; however, in multivariate analysis, the Sokal score was the only independent significant predictor of ETR in chronic myeloid leukemia.

There was a significant variance regarding microRNA-21 expression between studied CML patients & studied healthy controls, as mean values were higher in patients with a significant variance as the p value was lower than 0.05.

Actually, our study is in accordance with **Mirza et al. [12].** This research included hundred CML cases clinically confirmed by the presence of a Bcr/Abl transcript for CML cases & 100 healthy control subjects. The investigation detected microRNA-21 overexpression in CML cases, as well as a gradual increase in expression as the disease progressed. Before treatment, the expression of microrna-21 in chronic myeloid leukemia cases has been observed to be 9.22-fold higher than that in the control group. A significant difference (p-value less than 0.0001) was observed in the expression of miR-21 among cases, with values increasing in advanced stages such as the accelerated phase (10.30-fold), blast crisis (13.20-fold), & the chronic phase (7.16-fold).

Mean values of BCR-ABL after 6 months among TKI responders & non-responders showed a significant difference, as the p-value was 0.002.

Habib et al. [11] were consistent with our investigation regarding *BCR-ABL1* transcript level at six months, as our study demonstrated a significant variance with a p-value of 0.002 regarding the mean values of BCR-ABL after 6 months among TKI responders &non-responders being higher in non-responders, while BCR-ABL after 6 months became 1.34±0.46 in the TKI response group & 10.62±11.27 in the TKI resistance group, showing significant differences with a p-value of 0.002, which is reasonable.

Also, our predictive data showed that microRNA-21 had a statistically significant role in the prediction of CML disease. At a cutoff of 1.2 with 53% sensitivity & 88% specificity.

Numerous studies have shown that miR21, whose gene is located in chromosome 17q23.2c, is an oncomiR [**13]**, is essential in the proliferation & apoptosis of various tumors, & participates in the regulation of various downstream effectors correlated to tumors **[14].**

Our study showed that microRNA-21 had a statistically significant role in the therapeutic response to tyrosine kinase inhibitors. At a cutoff of 0.73 with 86% sensitivity & 66% specificity.

In agreement with **Alves et al.'s [13]** work, which aimed to correlate the expression levels of three microRNAs, microRNA-451, microRNAs-21, &microRNAs-26b, with the response to tyrosine kinase inhibitor management in CML cases, microRNA-451 levels at diagnosis have been significantly elevated in cases with an optimal response after six & twelve months of treatment. In contrast, cases that did not exhibit an optimal response exhibited the highest levels of micoRNAs-21. MicroRNAs-21& microRNAs-451 seem to be effective biomarkers of response, able to predict optimal tyrosine kinase inhibitor responders (p-value less than 0.05). They developed a predictive

model of the optimal response after 1 year of therapy by utilizing the combined profiles of both miRs. In predicting which cases obtain the optimal response, this investigation highlights the role of micriRNA-21 & microRNA-451 expression levels at the time of diagnosis.

There was no correlation between micro-RNA-21 expression & BCR-ABL after 6 months (p-value 0.465).

Conclusion

The miRNA-21 expression levels were significantly higher in CML cases compared to the controls (1.68±1.26, 0.90±0.22) (p-value, 0.0001). The expression levels of microRNA-21 in CML cases decreased after TTT, with TKI being higher in TKI-resistant patients. There was no correlation between micro-RNA 21 expression &BCR-ABL after 6 months of Imatinib (p-value 0.465). There was no association between micro-RNA 21 expression & the Sokal score (p-value 0.423).

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