AIM OF THE WORK

The aim of the present work is to study the possible value of micro RNA 451 (miR-451) in chronic myeloid leukemia (CML) as an early predictor for Imatinib Mesylate (IM) resistance in Egyptian patients.

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SUBJECTS

The present study was conducted on 60 subjects including 45 chronic myeloid leukemia (CML) patients who were recruited from the Hematology Unit of Alexandria Main University Hospital and 15 subjects as the control group.

The subjects were categorized into the following four groups:

- **Group (I):** 15 chronic phase adult CML patients. The diagnosis was based upon clinical evaluation, complete blood count (CBC) and the detection of BCR-ABL fusion gene by Real time Polymerase Chain Reaction (qPCR).
- **Group (II):** 15 Imatinib Mesylate (IM) responder CML patients.
- **Group (III):** 15 IM resistant CML patients, 3 months after treatment onset. The response and resistance to IM were scaled according to the 2014 National Cancer Comprehensive Network (NCCN) guidelines.[30]
- **Group (IV):** 15 age and sex matched apparently healthy subjects as the control group.

The study was conducted in accordance with the ethical guidelines of 1975 Declaration of Helsinki and was approved by the Local Ethics Committee of the Faculty of Medicine, University of Alexandria. An informed consent was obtained from all subjects included in the study.

Exclusion criteria:

Subjects who had any of the following conditions were excluded from the study: Acute and chronic infections, chronic illness (cardiac, respiratory, hepatic or renal), chronic alcoholism and other malignancies.

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METHODS

• All patients (groups I, II, and III) were subjected to the following:

I- Thorough history taking and complete clinical examination:

History taking and clinical examination of patients focused on symptoms and signs chronic myeloid leukemia (CML) which may include: huge splenomegaly, malaise, clinical evidence of leukostasis (High leukocyte level leading to vascular obstruction which induces tissue hypoxia) [(130)], joint pain, low-grade fever, susceptibility to infections, anemia, and thrombocytopenia with easy bruising.

II- BCR-ABL gene mutation detection in chronic myeloid leukemia patients.

The percentage of BCR-ABL transcript levels in CML patients were obtained from the routine monitoring of patients using Real time Polymerase Chain Reaction (qPCR) that is standardized within the frames of international standardization.[(131)]

III- Calculation of relative risk.

Sokal score was calculated at http://www.icsg.unibo.it/rrcalc.asp.

• All subjects (groups I,II,III, and IV) were subjected to the following:

I- Routine laboratory investigations including:

1. Complete blood count (CBC).[(132)]
2. Liver function tests including serum alanine aminotransferase (ALT) and serum aspartate aminotransferase (AST).[(133)]
3. Kidney function tests including serum urea and creatinine.[(134)]

II- Measurement of leucocytic level of micro-RNA 451 (miR-451) using qPCR.

- **Sample collection:**

Blood samples were obtained by venipuncture using sterile disposable plastic syringes. About 10 milliliters of venous blood were withdrawn aseptically after disinfection of the skin using 70% alcohol and betadine, and divided into the following:

- Two ml of blood were delivered into a vacutainer ethylene diamine tetra-acetic acid (EDTA) tube for CBC.

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- Three ml of blood were delivered into a vacutainer plain tube then centrifuged and the serum was separated. The separated serum was used for routine liver and kidney function tests.
- Two ml of blood were delivered into a vacutainer EDTA tube for BCR ABL analysis.
- Three ml of blood were delivered into a vacutainer EDTA tube miR-451 analysis.

- **Measurement of leukocyte level of miR-451:**

Reverse Transcription Quantitative Polymerase Chain Reaction (qRT-PCR) was employed in this study.

- Done for all patients and controls.
- Extraction of total RNA was done using Qiagen® miRNeasy Mini Kit.
- Quantification of miR-451 was performed using Applied Biosystems® TaqMan MicroRNA Assays in a two-step RT-PCR reaction:
- In the reverse transcription (RT) step, complementary deoxyribonucleic acid (cDNA) was reverse transcribed from total RNA samples using specific miRNA primers from the TaqMan MicroRNA Assays and reagents from the TaqMan® MicroRNA Reverse Transcription Kit.
- In the Polymerase Chain Reaction (PCR) step, PCR products were amplified from cDNA samples using the TaqMan MicroRNA Assay together with the TaqMan® Universal PCR Master Mix.

Procedural Overview:

1. Preparation of total RNA
2. Performing reverse transcription
3. PCR process
4. Analysis of the results.

Steps:

1. Preparation of total RNA (Using Qiagen miRNeasy Mini Kit)

- Three ml of blood human whole blood was mixed with 15 ml of erythrocyte lysis buffer (Buffer EL) in an appropriately sized tube. The mixture was incubated for 10–15 min on ice. Mixing by brief vortexing two times during incubation was performed. The cloudy suspension became translucent during incubation, indicating lysis of erythrocytes.
- The mixture was centrifuged at 400 x g for 10 min at 4°C and the supernatant was completely removed and discarded and the leukocytes formed a pellet after centrifugation. For pelleted cells, the pellet was loosened by flicking the tube thoroughly.
- 700 µl of QIAzol Lysis Reagent was added to the pellet. Mixing was achieved by vortexing or pipetting.
- The tube containing the homogenate was placed on the benchtop at room temperature (15–25°C) for 5 min. This step promoted dissociation of nucleoprotein complexes.

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- 140 µl of chloroform was added to the tube containing the homogenate and the tube was capped securely.
- Vigorous vortexing of the tube was performed for 15 s. Thorough mixing was important for subsequent phase separation.
- The tube containing the homogenate was placed on the benchtop at room temperature for 2–3 min. Then, the homogenate was centrifuged for 15 min at 12,000 x g at 4°C.
- After centrifugation, the sample separated into 3 phases: an upper, colorless, aqueous phase containing RNA; a white interphase; and a lower, red, organic phase. The volume of the aqueous phase was approximately 350 µl.
- The upper aqueous phase was transferred to a new collection tube. 1.5 volumes (usually 525 µl) of 100% ethanol was added and thorough mixing by pipetting up and down several times was performed.
- 700 µl of the sample, was pipetted into an RNeasy Mini spin column in a 2 ml collection tube.
- Centrifugation was performed at ≥8000 x g (≥10,000 rpm) for 15 s at room temperature (15–25°C). The flow-through was discarded.
- The preceeding step was repeated using the remainder of the sample.
- 700 µl Buffer RWT was added to the RNeasy Mini spin column, and centrifuged for 15 s at ≥8000 x g (≥10,000 rpm) to wash the column. The flow-through was discarded. (Guanidine salt and ethanol are important ingredients in Buffer RWT. Ethanol was added prior to the first use of the kit as buffer RWT is supplied as a concentrate)
- 500 µl Buffer RPE was pipetted onto the RNeasy Mini spin column and centrifuged for 15 s at ≥8000 x g (≥10,000 rpm) to wash the column. The flow-through was discarded. (Buffer RPE is supplied as a concentrate, ethanol was therefore added by prior to the first use of the kit. It functions to wash membrane-bound RNA)
- Another 500 µl Buffer RPE was added to the RNeasy Mini spin column and centrifuged for 2 min at ≥8000 x g (≥10,000 rpm) to dry the RNeasy Mini spin column membrane.
- The RNeasy Mini spin column was placed into a new 2 ml collection tube and the old collection tube with the flow-through was discarded.
- Centrifugation in a microcentrifuge was then performed at full speed (≥14,000 rpm) for 1 min. This step helped to eliminate any possible carryover of Buffer RPE or if residual flow-through remained on the outside of the RNeasy Mini spin column.
- The RNeasy Mini spin column was transferred to a new 1.5 ml collection tube. 30–50 µl RNase-free water was pipetted directly onto the RNeasy Mini spin column membrane and centrifuged for 1 min at ≥8000 x g (≥10,000 rpm) to elute the RNA.

2. Performing reverse transcription

Single-stranded cDNA was synthesized from total RNA samples using the TaqMan® MicroRNA Reverse Transcription Kit. The process involved the following procedures:

a. Preparing the RT master mix

- RT master mix was prepared using the TaqMan MicroRNA Reverse Transcription Kit components.
- In a polypropylene tube, RT master mix was prepared on ice by scaling the volumes listed below to the desired number of RT reactions.

Table. (4): Table for reverse transcription master mix preparation.

Component	Master mix volume per 15-µL reaction (µL)
100mM dNTPs (with dTTP)	0.15
MultiScribe™ Reverse Transcriptase, 50 U/µL	1.00
10× Reverse Transcription Buffer	1.50
RNase Inhibitor, 20 U/µL	0.19
Nuclease-free water	4.16
Total volume	**7.00**

b. Preparing the RT reaction plate

- For each 15-µL RT reaction, RT master mix was combined with total RNA in the ratio of: 7 µL RT master mix to 5 µL total RNA.
- Mixing and Centrifugation were performed to bring the solution to the bottom of the tube.
- For each 15-µL RT reaction, 12.0 µL of RT master mix containing total RNA were dispensed into a 0.2-mL polypropylene reaction tube. (This is the RT reaction tube). Then, 3 µL of RT primer (tube labeled RT Primer) were transferred from each assay set into the corresponding RT reaction tube or plate well.
- The tube was sealed and gentle mixing was performed. Centrifugation to bring solution to the bottom of the tube was done.
- The tube was incubated on ice for 5 min and kept on ice until we were ready to load the thermal cycler.

c. Performing Reverse Transcription

- The following parameter values were used to program the thermal cycler (Primus 25 advanced, PEQLAB, United Kingdom):

Table (5): Table for reverse transcription program

Step	Time	Temperature °C
Hold	30 minutes	16
Hold	30 minutes	42
Hold	5 minutes	85
Hold	∞	4

- The reaction volume was set to 15.0 µL. The reaction tube or plate was loaded into the thermal cycler.
- Then the reverse transciption run was started.

- After the RT run, the RT reaction was stored at − 15 to − 25 °C, if PCR amplification was not to be started immediately.

3. Real time Polymerase Chain Reaction:

Real time Polymerase Chain Reaction was performed using Applied Biosystems StepOne™ Real-Time PCR System.

Fig.(10) Applied Biosystems StepOne™ Real-Time PCR System.

Real time PCR included the following procedures:

a. Preparing the reaction.

- The volumes listed below were scaled to the appropriate number of RT reactions.

Table (6): Table for polymerase chain reaction master mix preparation.

Reagent	Master Mix Volume for One 20 µL Reaction (µL)
TaqMan® Universal Master Mix II, no UNG	10.00
Nuclease-free water	7.67
Total Volume	17.67

- Mixing and centrifugation were done to bring solution to the bottom of the tube.
- 17.67 µL of the PCR master mix/water mixture per 20-µL PCR reaction were added into a polypropylene tube (the PCR reaction tube).
- 1.0 µL of 20✕ TaqMan MicroRNA Assay mix (labeled Real Time) were transferred into the PCR Reaction tube.
- Then, 1.33 µL of the RT product were transferred from the RT reaction tube into the PCR reaction tube.
- Mixing and centrifugation were done to bring solution to the bottom of the plate.

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- The PCR reaction plate was prepared by dispensing 20 µL of the complete PCR master mix (including primer and RT product) into each of four wells.
- The plate was sealed with an optical adhesive cover.
- The TaqMan® Universal Master Mix II, with or no UNG contained
  - AmpliTaq Gold® DNA Polymerase, UP (Ultra Pure)
  - dNTPs with dUTP
  - ROX™ Passive Reference
  - Optimized buffer components.

b. Setting up the plate document.

- Applied Biosystems StepOne™ Real-Time PCR System was employed.
- In the real-time PCR system software, an experiment or plate document was created using the following parameters:
  - Run Mode: Standard
  - Sample Volume: 20 µL
  - Thermal Cycling Conditions:

Table (7): Table for polymerase chain reaction program.

Step	AmpErase® UNG activity	Enzyme Activation	PCR	
			CYCLE (40 cycles)	
	HOLD	HOLD	Denature	Anneal/extend
Temperature	50 °C	95 °C	95 °C	60 °C
Time	2 minutes	10 minutes	15 seconds	60 seconds

c. Running the plate.

To run the plate, the plate document that corresponds to the reaction plate was opened. Then, the reaction plate was loaded into the instrument and the run was started.

4. Analysis of the results

The general process for analyzing the data from gene expression assays involved the following procedures:

a. Viewing the amplification plots.
b. Setting the baseline and threshold values.
c. Relative gene expression data were analysed using the $2^{-\Delta\Delta CT}$ Method.[135]

Assay normalization with TaqMan® endogenous controls:

Using the comparative CT method, endogenous control (miR-30c) was used to normalize the expression levels of target (miR-451).

The fold change in expression of the target gene relative to the internal control gene was studied. The Ct (cycle threshold) data were calculated using $2^{-\Delta\Delta CT}$ method.[135]

Statistical analysis of the data.

Data were fed to the computer and analyzed using IBM SPSS software package version 20.0.[136] Qualitative data were described using number and percent. Quantitative data were described using range (minimum and maximum), mean, standard deviation and median. Comparison between different groups regarding categorical variables was tested using Chi-square test. When more than 20% of the cells have expected count less than 5, correction for chi-square was conducted using Monte Carlo correction. The distributions of quantitative variables were tested for normality. If it reveals normal data distribution, parametric tests was applied. If the data were abnormally distributed, non-parametric tests were used. For normally distributed data, comparison between more than two population were analyzed F-test (ANOVA) to be used and Post Hoc test (Tukey. For abnormally distributed data, Kruskal Wallis test was used to compare between different groups and pair wise comparison was assessed using Mann-Whitney test. Significance test results are quoted as two-tailed probabilities. Significance of the obtained results was judged at the 5% level.[137]

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RESULTS

This study included 60 subjects allocated into four groups:

Group I: 15 chronic phase adult CML patients.

Group II: 15 IM responder CML patients.

Group III: 15 IM resistant CML patients.

Group IV: 15 age and sex matched apparently healthy subjects (control group).

I. The demographic data:

As shown in table 8, figures 11 and 12, there were no statistically significant differences between the studied groups regarding the subjects' gender and age.

Table (8): Comparison between the studied groups according to demographic data

Parameter	Group I (n = 15)		Group II (n = 15)		Group III (n = 15)		Group IV (Control) (n = 15)		Test of sig.	p
	No.	%	No.	%	No.	%	No.	%		
Gender										
Male	7	46.7	10	66.7	7	46.7	6	40.0	χ^2= 2.400	0.494
Female	8	53.3	5	33.3	8	53.3	9	60.0		
Age(years)										
Mean ± SD.	41.0 ± 13.20		45.60 ± 11.53		44.40 ± 11.33		39.0 ± 12.77		F= 0.927	0.434

χ^2: Chi square test
F: F test (ANOVA)

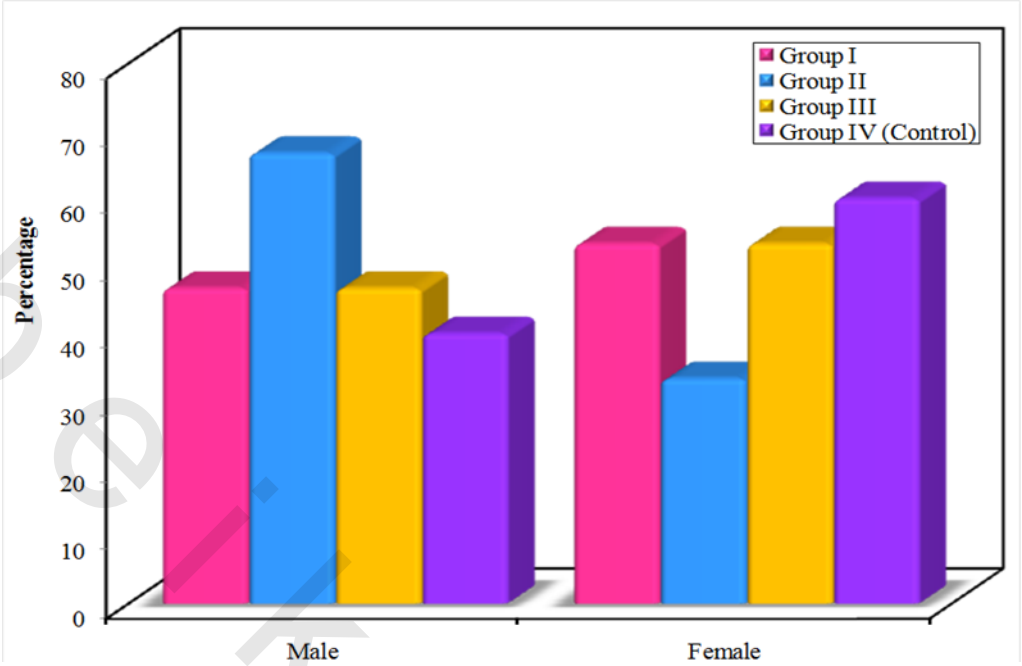

Figure (11): Comparison between the studied groups according to gender

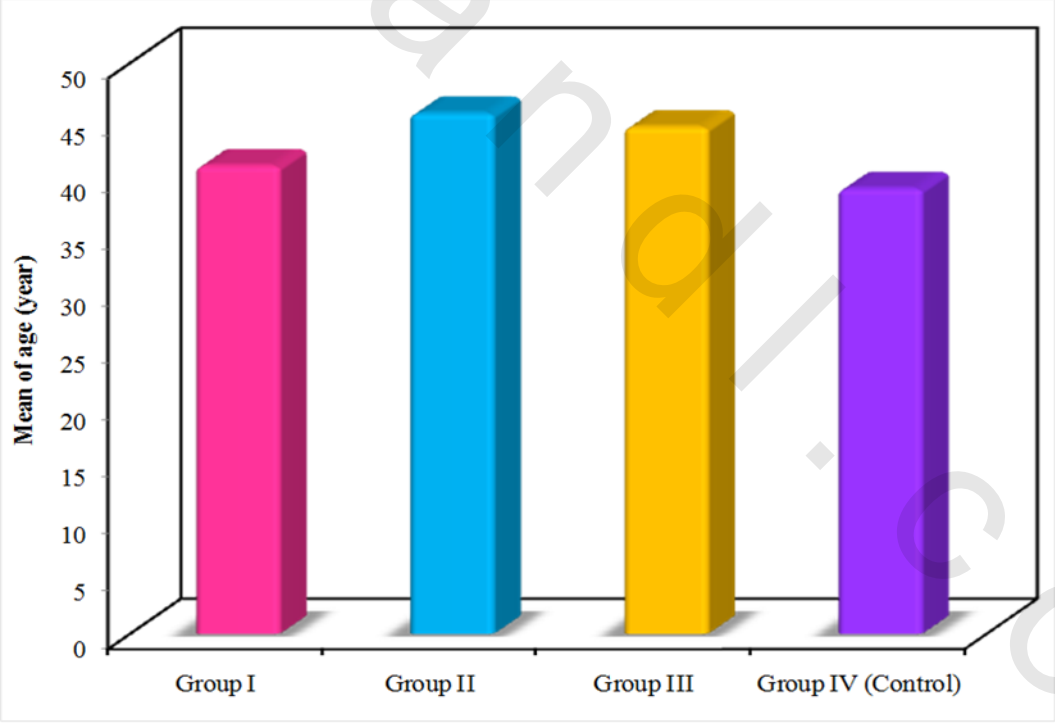

Figure (12): Comparison between the studied groups according to age

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II. Complete blood count:

The results shown in table 9 demonstrate the comparison between the studied groups according to complete blood count (CBC) parameters as follows:

1. ***Hemoglobin concentration (Hb)(g/dl):*** There was a significant reduction in hemoglobin concentration in group I compared to group II, group III and group IV ($p< 0.001$).
2. ***Platelet count ($x10^9$/l):*** A statistically significant decrease in platelet count was shown in groups I compared with group II, group III and group IV ($p< 0.001$).
3. ***White blood cell count ($x10^9$/l):*** There was a significant increase in WBC count in group I compared to group II, group III and group IV ($p< 0.001$).

Table (9): Comparison between the studied groups according to complete blood count.

Parameter	Group I (n = 15)	Group II (n = 15)	Group III (n = 15)	Group IV (Control) (n = 15)	F	p
Hb value (g/dl)						
Mean ± SD.	10.88 ± 1.07	14.46 ± 1.27	14.57 ± 1.49	14.13 ± 1.32	27.620*	<0.001
$p_{Con.}$	<0.001*	0.895	0.790			
Sig. bet. grps	p_1<0.001*, p_2<0.001*, p_3= 0.996					
Platelet count ($x10^9$/L)						
Mean ± SD.	174.87 ± 31.83	320.67 ± 52.37	342.27 ± 133.13	308.40 ± 63.57	13.495*	<0.001*
$p_{Con.}$	<0.001*	0.975	0.654			
Sig. bet. grps	p_1<0.001*, p_2<0.001*, p_3= 0.880					
WBC count ($x10^9$/L)						
Mean ± SD.	29.24 ± 6.95	7.18 ± 20.02	7.64 ±1.888	7.75 ± 1.60	121.025*	<0.001*
$p_{Con.}$	<0.001*	0.977	1.000			
Sig. bet. grps	p_1<0.001*, p_2<0.001*, p_3= 0.988					

F: F test (ANOVA)
p_{Con}: p value for Post Hoc test (Tukey) for comparing between control and each other groups
p_1: p value for Post Hoc test (Tukey) for comparing between group I and group II
p_2: p value for Post Hoc test (Tukey) for comparing between group I and group III
p_3: p value for Post Hoc test (Tukey) for comparing between group II and group III
*: Statistically significant at $p \leq 0.05$

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III. The prognostic scoring system (Sokal score):

No statistically significant differences (p= 0.548) were found between CML cases according to Sokal score as shown in table 10.

Table (10): Comparison between CML cases according to Sokal score.

Parameter	Group I (n = 15)		Group II (n = 15)		Group III (n = 15)		Test of sig.	p
	No.	%	No.	%	No.	%		
Sokal score								
Low	2	13.3	0	0.0	1	6.7		
Intermediate	8	53.3	9	60.0	6	40.0	χ^2= 2.601	^{MC}p=0.548
High	5	33.3	6	40.0	8	53.3		

χ^2: Value for chi square
MC: Monte Carlo test

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IV. BCR-ABL%:

Results presented in table 11 showed no statistically significant difference between groups I, II and II regarding the median BCR-ABL% at diagnosis (p= 0.201).

In group I, follow up BCR-ABL% had a median value of 7.90%. In group II, it was 0.20%. However, in group III, the median value of BCR-ABL% was 67.0%.

The median follow up BCR-ABL% was significantly higher in group I compared to group II (p= 0.034). Also it was significantly higher in group III than in group I (p< 0.001) and group II (p< 0.001).

Table (11): Comparison between the studied groups according to BCR-ABL% at diagnosis and BCR-ABL% after treatment.

Parameter	Group I (n = 15)	Group II (n = 15)	Group III (n = 15)	Test of sig.	p
BCR-ABL% at diagnosis					
Min. – Max.	2.0 – 100.0	29.0 – 100.0	38.0 – 100.0	$^{KW}\chi^2$= 3.211	0.201
Median	64.0	73.0	87.0		
BCR-ABL% on follow up					
Min. – Max.	0.0 – 100.0	0.0 – 9.0	10.0 – 100.0	$^{KW}\chi^2$= 26.021*	<0.001*
Median	7.90	0.20	67.0		
Sig. bet. grps	p_1= 0.034*, p_2<0.001*, p_3<0.001*				

χ^2: Value for chi square
MC: Monte Carlo test
$^{KW}\chi^2$: Chi square for Kruskal Wallis test
p_1 : p value for Mann Whitney test for comparing between group I and group II
p_2 : p value for Mann Whitney test for comparing between group I and group III
p_3: p value for Mann Whitney test for comparing between group II and group III
*: Statistically significant at $p \leq 0.05$

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V. Micro RNA 451 level in the studied groups

As shown in table 12 and figure 13 the median value of miR-451 level was 2.30, while in group II, it was 8.94. In group III, the median value of miR-451 was 6.72. However, in group IV, miR-451 median level was 3.79.

There was a significant decrease in the median miR-451 in group I compared with group II ($p< 0.001$), group III ($p= 0.007$) and to the control group IV ($p= 0.002$).

However, group II showed a significant increase in the median level of miR-451 in comparison with group III ($p= 0.046$) and the control group IV ($p< 0.001$).

Table (12): Comparison between miR-451 level in the studied groups.

Parameter	Group I (n = 15)	Group II (n = 15)	Group III (n = 15)	Group IV (Control) (n = 15)	$^{KW}\chi^2$	p
miR-451						
Min. – Max.	0.40 – 121.90	3.65 – 23.10	0.02 – 11.63	2.98 – 5.36	29.153*	<0.001*
Median	2.30	8.94	6.72	3.79		
$p_{Con.}$	0.002*	<0.001*	0.010*			
Sig. bet. grps	$p_1<0.001^*$, $p_2= 0.007^*$, $p_3= 0.046^*$					

$^{KW}\chi^2$: Chi square for Kruskal Wallis test
p_{Con}: p value for Mann Whitney test for comparing between control and each other groups
p_1 : p value for Mann Whitney test for comparing between group I and group II
p_2 : p value for Mann Whitney test for comparing between group I and group III
p_3 : p value for Mann Whitney test for comparing between group II and group III
*: Statistically significant at $p \leq 0.05$

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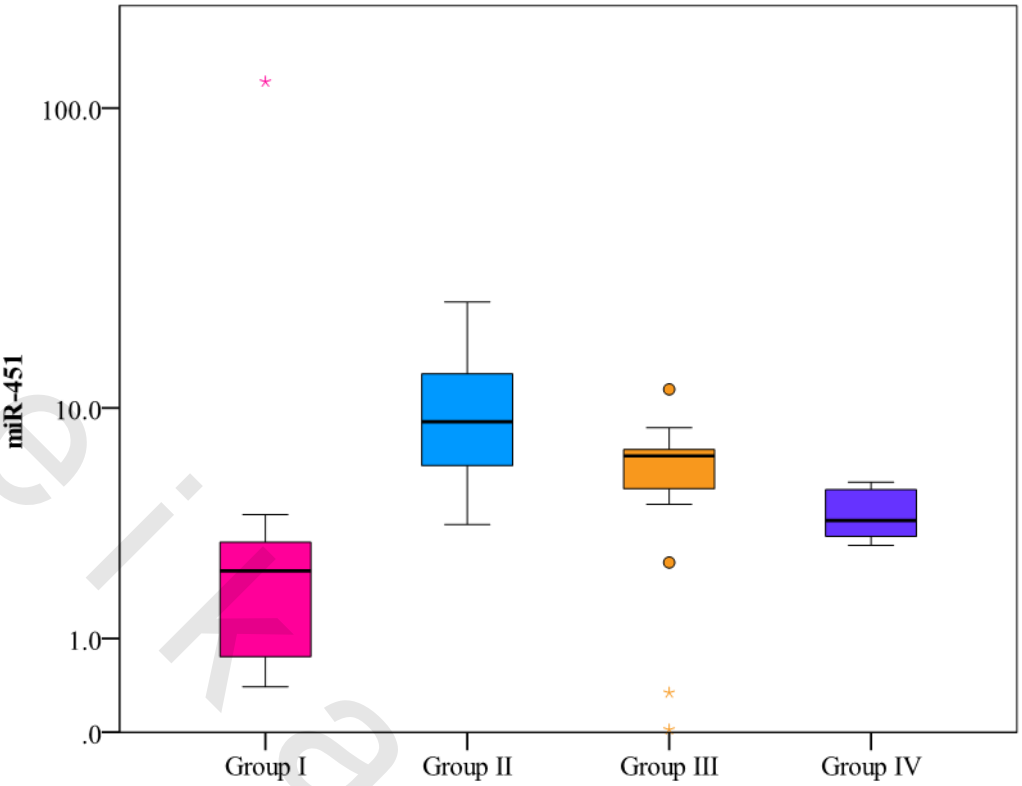

Figure (13): miR-451 in the studied groups

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VI. Comparison between the studied groups according to miR-451 expression:

As shown in table 13, miR-451 was down-regulated in leucocytes of 12 (80%) patients, 4 (26.7%) patients and 5 (33.3%) in groups I, II and III respectively while it was up-regulated in 3 (20%) patients, 11 (73.3%) and 10 (66.7%) in groups I, II and III respectively.

Table (13): Comparison between the studied groups according to miR-451 expression

Parameter	Group I (n = 15)		Group II (n = 15)		Group III (n = 15)	
	No.	%	No.	%	No.	%
miR-451 expression						
Up-regulation	3	20.0	11	73.3	10	66.7
Down-regulation	12	80.0	4	26.7	5	33.3

VII. The relation between gender and miR-451 level:

Statistical analysis presented in table 14 showed no significant relation between gender and miR-451 in the four studied groups (p= 0.260)

Table (14): Relation between gender and miR-451 level in the studied groups.

Parameter	Gender		Z	P
	Male (n = 24)	Female (n = 21)		
miR-451				
Min. – Max.	0.40 – 23.10	0.02 – 121.90	1.126	0.260
Median	6.30	5.79		

Z: Z for Mann Whitney test

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VIII. Relation between age and miR-451:

No significant relation was found between age below and above 50 years and miR-451 in the studied groups (p= 0.417), as shown in table 15.

Table (15): Relation between age and miR-451 in the studied groups

Parameter	Age		Z	p
	<50 (n = 27)	≥50 (n = 18)		
miR-451				
Min. – Max.	0.02 - 121.90	0.40 - 13.68	0.811	0.417
Median	6.72	5.94		

Z: Z for Mann Whitney test

IX. Relation between Sokal score and miR-451

As shown in table 16 statistical analysis showed no significant relation between Sokal score and miR-451 in CML cases (p= 0.603)

Table (16): Relation between Sokal score and miR-451 in in CML cases

Parameter	Sokal score			$^{KW}\chi^2$	p
	Low (n = 3)	Intermediate (n = 23)	High (n = 19)		
miR-451					
Min. – Max.	0.90 – 7.94	0.34 – 121.90	0.02 – 13.68	1.013	0.603
Median	4.0	6.72	5.89		
p	$p_1 = 0.547$, $p_2 = 0.811$, $p_3 = 0.363$				

$^{KW}\chi^2$: Chi square for Kruskal Wallis test
p_1: p value for Mann Whitney test for comparing between low and intermediate
p_2: p value for Mann Whitney test for comparing between low and high
p_1: p value for Mann Whitney test for comparing between intermediate and high

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X. Relation between miR-451 expression with BCR-ABL% at diagnosis and follow up in groups I,II,II

In group I as shown in table 17 and figure 15, the relation between miR-451 expression was statistically significant with BCR-ABL % at diagnosis (p= 0.021), while no significant relation was shown with BCR-ABL % on follow up (p= 0.060).

In groups II and III, miR-451 expression showed a statistically significant relation with BCR-ABL % on follow up (p= 0.003 and p= 0.002) respectively while, no significant relation was shown with BCR-ABL % at diagnosis (p= 0.078 and 0.086) respectively

Table (17): Relation between miR-451 expression with BCR-ABL% at diagnosis and follow up in groups (I,II,III)

Parameter	miR-451 expression		Z	p
	Up-regulation (n = 3)	Down-regulation (n = 12)		
Group I				
BCR-ABL% diagnosis				
Min. – Max.	2.0 - 38.0	14.0 – 100.0	2.309*	0.021*
Median	8.0	67.50		
BCR-ABL% follow up				
Min. – Max.	0.0 – 5.0	0.0 – 100.0	1.883	0.060
Median	0.0	8.50		
Group II				
BCR-ABL% diagnosis	**(n = 11)**	**(n = 4)**		
Min. – Max.	29.0 -100.0	64.0 – 100.0	1.764	0.078
Median	67.0	94.0		
BCR-ABL% follow up				
Min. – Max.	0.0 – 6.0	7.0 – 9.0	2.969*	0.003*
Median	0.0	8.0		
Group III				
BCR-ABL% diagnosis	**(n = 10)**	**(n = 5)**		
Min. – Max.	38.0 – 100.0	69.0 – 100.0	1.718	0.086
Median	70.0	93.0		
BCR-ABL% follow up				
Min. – Max.	10.0 - 72.0	75.0 – 100.0	3.062*	0.002*
Median	51.50	88.0		

Z: Z for Mann Whitney test
*: Statistically significant at $p \leq 0.05$

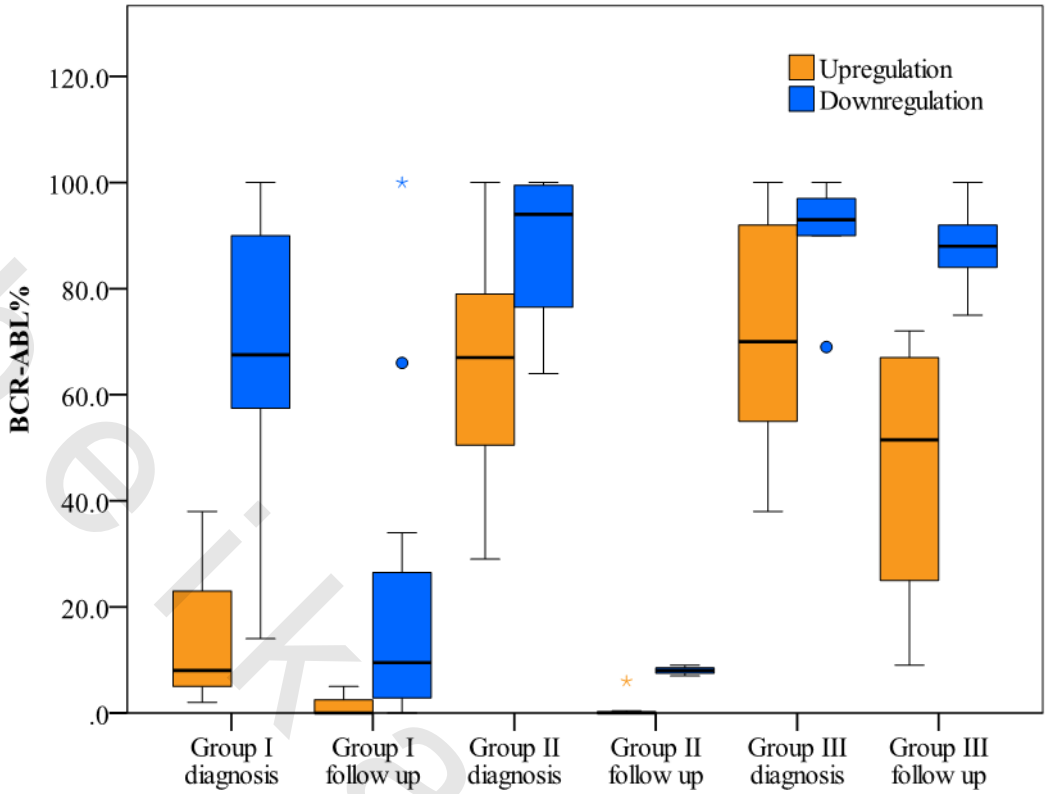

Figure (14): Relation between miR-451 expression with BCR-ABL% at diagnosis and follow up in groups (I,II,III)

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XI. Correlation between miR-451 with age, CBC and BCR-ABL

The statistical correlations between miR-451 and other studied parameters in patients of the different groups are presented in table 18, figures 15 and 16 and showed the following:

In group I miR-451 level showed a positive significant correlation with platelet count (p= 0.023) and a significant negative correlation with BCR-ABL% at diagnosis (p= 0.019).

Group II showed a significant negative correlation between miR-451 level with BCR-ABL% (p= 0.032) on follow up

However, group III showed no significant correlations between miR-451 level and any of the studied parameters.

Table (18): Correlation between miR-451 with age, CBC and BCR-ABL

Parameter		miR-451		
		Group I	Group II	Group III
Age	r_s	-0.501	-0.113	0.022
	p	0.057	0.689	0.939
Hb value (g/dl)	r_s	-0.076	0.082	0.004
	p	0.788	0.770	0.990
Platelets($10\times^{9}$/L)	r_s	0.580*	0.325	-0.286
	p	0.023*	0.237	0.302
WBC count ($10^{\times 9}$/L)	r_s	-0.059	-0.368	-0.118
	p	0.834	0.177	0.676
BCR-ABL% diagnosis	r_s	-0.597*	0.025	-0.134
	p	0.019*	0.929	0.634
BCR-ABL% follow up	r_s	-0.432	-0.554*	-0.311
	p	0.108	0.032*	0.260

r_s: Spearman coefficient
*: Statistically significant at $p \leq 0.05$

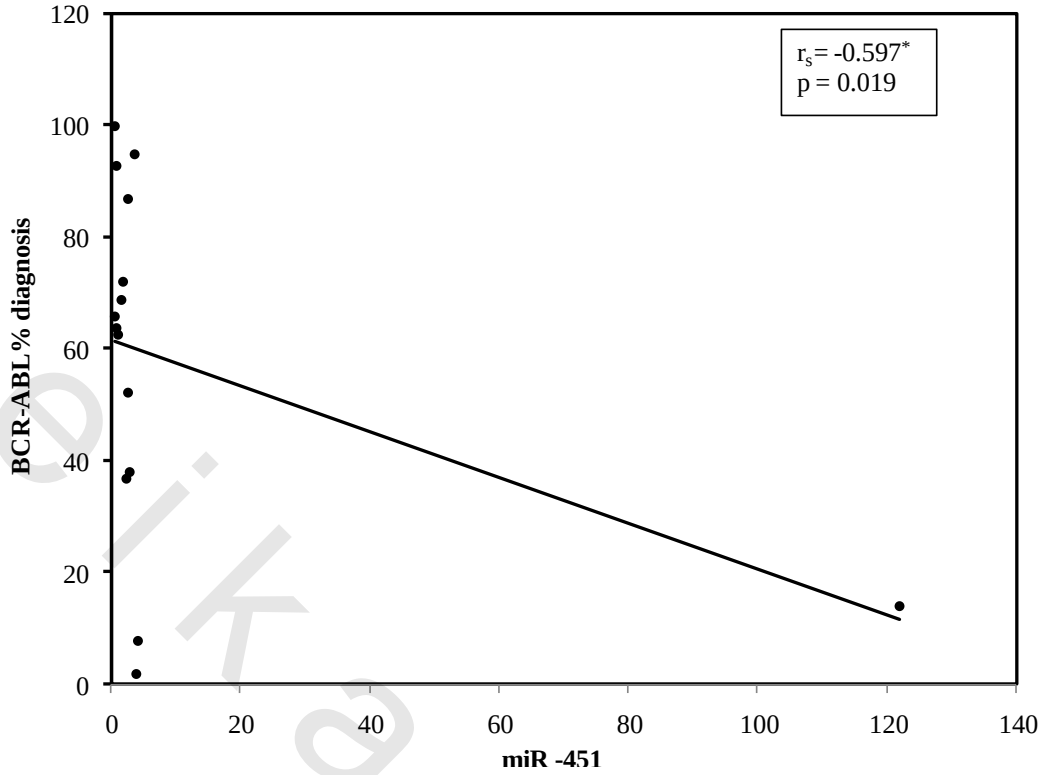

Figure (15): Correlation between miR-451 with BCR-ABL% diagnosis in group I

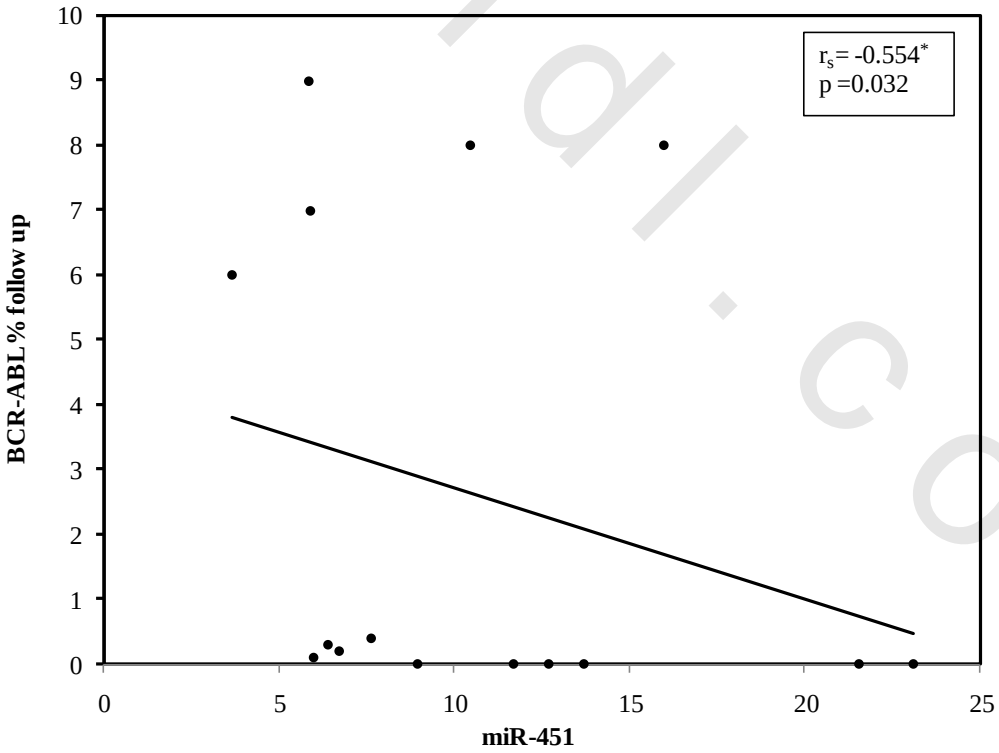

Figure (16): Correlation between miR-451 with BCR-ABL% follow up in group II

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XII. Sensitivity and specificity of for miR-451 as a prognostic marker for the CML.

The performance of miR-451 as a prognostic marker for CML have been determined by plotting a receiver-operating characteristic (ROC) curve shown in figure 17. The Area Under Curve (AUC) was found to be 0.677 (p= 0.044).

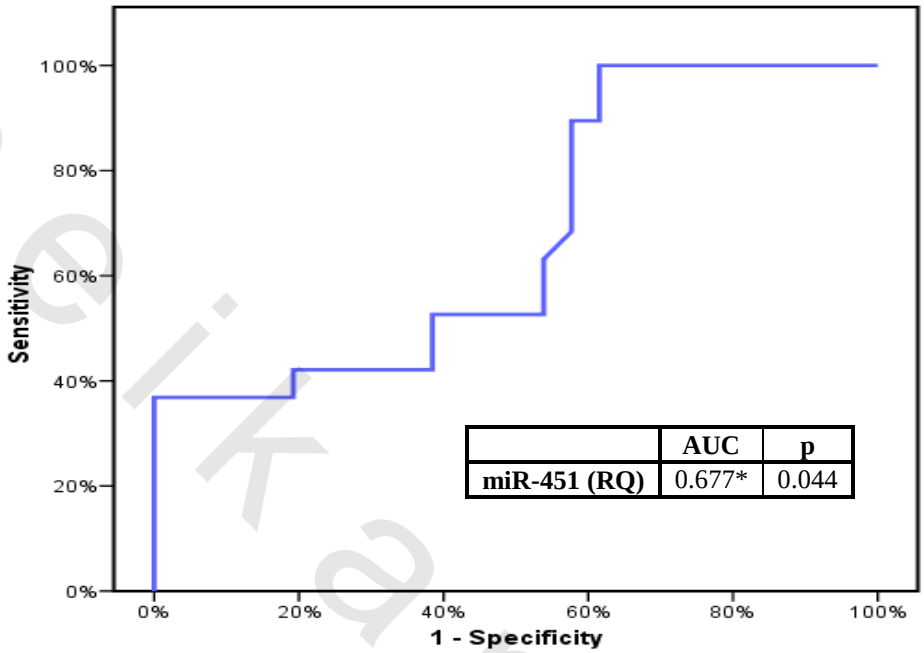

Figure (17): ROC curve for miR-451 and BCR-ABL% follow up in cases group (I+II+III) (n = 45)

As shown in table 19, the sensitivity of miR-451 in detecting CML to be 42.11% while its specificity has been shown to be 80.77% at the cut-off value of 2.51. The positive predictive (PPV) and the negative predictive value (NPV) had been estimated to be (61.54) and (65.63) respectively.

Table (19): Agreement (sensitivity, specificity and accuracy) for miR-451 BCR-ABL% follow up in cases group (I+II+III) (n = 45)

		BCR-ABL% follow up		Sensitivity	Specificity	PPV	NPV	Accuracy
		<10	≥10					
miR-451	>2.51	21	11	42.11	80.77	61.54	65.63	64.44
	≤2.51	5	8					

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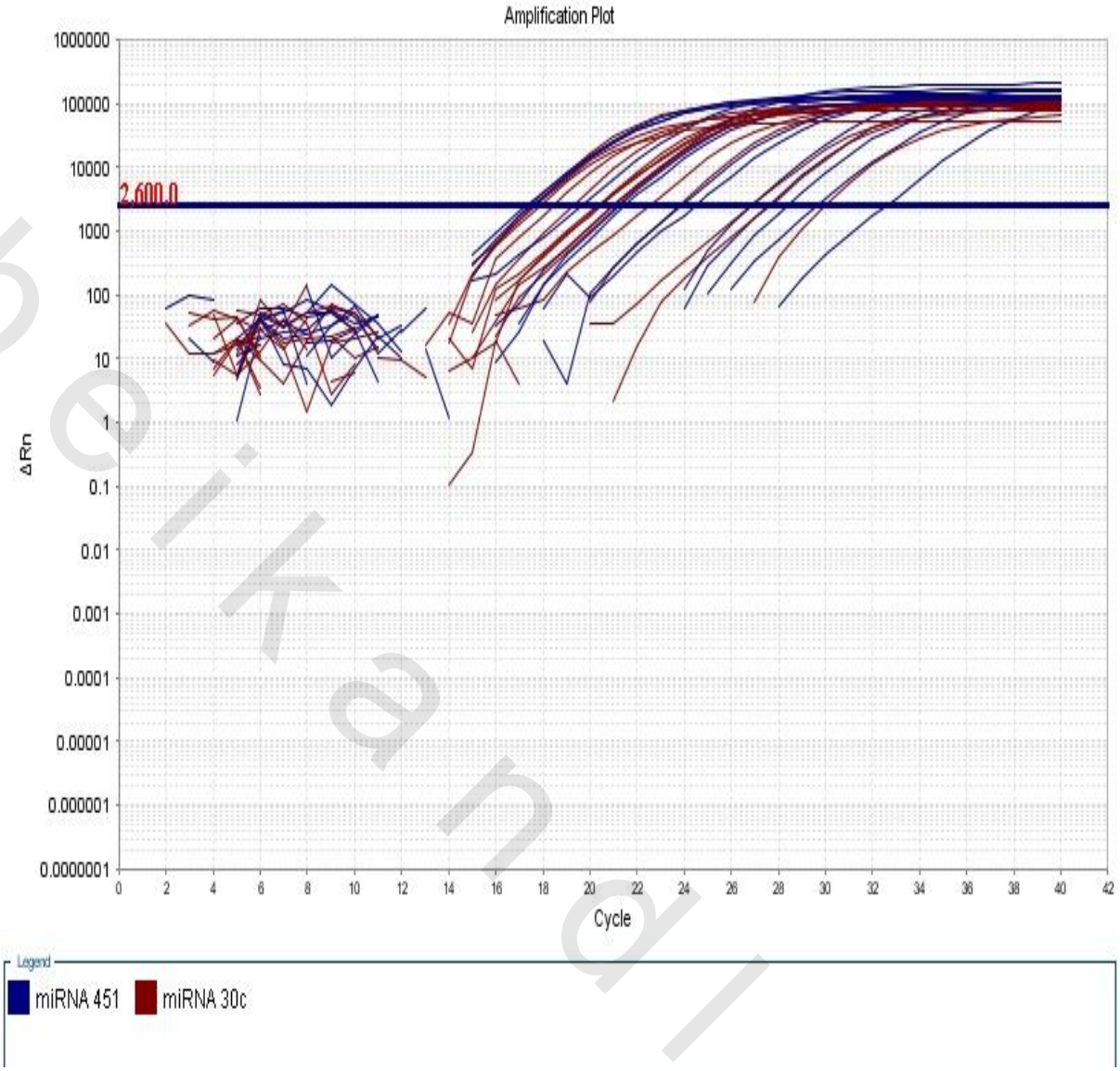

Figure (18): Amplification plot of miR-451 and miR-30c.

The amplification plot view of the target miRNA (miR-451) and the reference miRNA (miR-30c) showing a plot of amplification cycles (on the X axis) versus fluorescence units (on the Y axis)

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