DISCUSSION

Imatinib Mesylate (IM) is used as the first line tyrosine kinase inhibitor (TKI) for management of all newly diagnosed cases of CML. It acts by inhibiting the tyrosine kinase activity of BCR-ABL. In this regard, IM was one of the first cancer therapies to show the potential for such targeted action, and is often cited as a paradigm for research in cancer therapeutics.[28]

Treatment by IM could induce nearly complete hematologic and cytogenetic remissions in most patients with CML. However, despite its high efficiency, still some patients develop resistance to the drug. In chronic phase, this response failure was shown in about 25% of cases. Therefore, IM resistance represents the major limit to its targeted therapy efficacy.[27]

However, it is currently impossible to predict whether a patient will develop resistance to IM or not. The existence of second generation TKIs (Dasatinib and Nilotinib), which are effective in patients with IM resistance, makes the identification of early predictors of resistance to IM an important goal in treatment of CML.[31]

The past decade had witnessed an explosion of knowledge regarding microRNAs (miRNAs) and their roles in both physiological and pathological contexts. Multiple studies on miRNAs and their target mRNAs in cancer had demonstrated that some miRNAs act as oncogenes and some others as tumor suppressor genes.[40,60]

As the important roles of miRNAs in cancer are being discovered, they have been described as potentially valuable noninvasive prognostic or diagnostic cancer biomarkers in order to monitor molecular changes in tumors, which may assist in earlier diagnosis and in the selection of the best possible treatment for individual cancer patients.[61] Furthermore, therapeutic strategies involving re-introduction of miRNAs lost in cancer or inhibition of oncogenic miRNAs are rapidly being developed.[84]

Accumulating evidence indicated that miRNAs play crucial roles in myeloid development and leukemogenesis. In addition, the implication of miRNAs in the regulation of important biological processes altered in CML; such as cell cycle, apoptosis, and adhesion established these small RNA molecules as potential players in CML pathogenesis.[31]

Several previous works had reported aberrant miRNA expression in CML. Despite the different laboratory methods employed by these studies for microRNA expression profiling, significant overlap in the findings of these works exists, highlighting that studying miRNAs is crucial in understanding the aberrant CML hematopoiesis as well as the treatment resistance.[138]

In the first work concerning miRNA in CML, Venturini et al 2007,[97] had demonstrated enhanced expression of the miR-17-92 polycistronic cluster in CML CD34+ cells. In another study, Agirre et al 2008,[78] detected an abnormal miRNA expression profile in mononuclear and CD34+ cells from patients with CML compared with healthy controls. Moreover, Bueno et al 2008,[98] reported the hypermethylation of miR-203 in CML and also identified ABL as a putative miR-203 target.

With the objective of identifying a potential miRNA expression signature associated with IM resistance in CML, Enériz et al 2009,[31] identified 19 miRNAs differentially expressed between IM resistant and IM responder samples. Furthermore, the possible targets for these miRNAs were several membrane transporters that belong to the ATP binding cassette (ABC) super family of trans-membrane transporters, which had been implicated in resistance to chemotherapy.

Moreover, Flamant et al 2010,[101] used microarray analysis to study miRNA expression in newly diagnosed CML patients before and within the first two weeks of IM therapy. An increased expression of miR-150 and miR-146a, and reduced expression of miR-142-3p and miR-199b-5p after two weeks of IM therapy was reported. This study therefore concluded that IM treatment of CML patients rapidly normalizes the characteristic miRNA expression profile, suggesting that miRNAs may serve as a novel clinically useful biomarker in this disease.

Additional integrated microRNA expression profiling was provided by Machová et al 2011,[100] The study employed microarrays to identify differential expression profiles of miRNAs at different phases of CML. Expression deregulation was confirmed by Real time Polymerase Reaction (qPCR) and target genes of these miRNAs were identified to encode proteins that are involved in cell cycle and growth regulation as well as several key signaling pathways such as of mitogen activated kinase-like protein (MAPK), epidermal growth factor receptor (EGFR), transforming growth factor beta (TGFB1) and tumor suppressor protein p53.[100]

Taken together, these findings strongly suggested that an understanding of the molecular biology of CML will require consideration of both miRNomes and mRNA transcriptomes.[139] In the future, microRNA-based therapy is planned to be one of the approaches in CML management.[138]

The present study was designed to validate the value of microRNA 451 (miR-451) in CML as an early predictor for IM resistance in Egyptian patients. The study employed qPCR technique to investigate the expression of miR-451 in fifteen newly diagnosed CML patients (group I), fifteen IM responder CML patients (group II), fifteen IM resistant CML patients (group III) and fifteen healthy subjects of matched age and sex as a control group (group IV). The response to IM was defined as BCR-ABL transcript level < 10% after 3 months of first-line IM therapy.[30] The four studied groups were perfectly matched regarding gender and age.

Data from various studies indicated that the aberrant expression of miR-451 was associated with response to antitumor therapies in different human cancers. For example up-regulation of miR-451 was shown to inhibit proliferation and to trigger apoptosis of non-small cell lung cancer (NSCLC) [117] Up-regulation of miR-451 could also sensitize NSCLC A549 cells to cisplatin.[118]

Godlewski et al 2010,[123,124] showed that miR-451 combined with IM had a cooperative effect on growth inhibition of glioblastoma stem cells. Similarly, higher expression of miR-451 and miR-15b in pretreatment osteosarcoma samples correlated with subsequent positive response to neoadjuvant chemotherapy.[122] In gastric cancer patients, low miR-451 expression had been associated with higher risk of disease-relapse and was

also involved in radiation response.[120] Aberrant miR-451 could also confer drug resistance by promoting the self-renewal ability of colon cancer cells.[121]

MicroRNAs are known to have the ability to reduce protein expression by causing translational inhibition or degradation of the target mRNA, and their inhibition could lead to an increase in target protein expression. Interestingly, Zhu et al 2008,[140] reported that the expressions of miR-451 were increased in multidrug resistant (MDR) cancer cells, and its inhibition resulted in reductions of both permeability glycoprotein (P-gp) also known as multidrug resistance protein 1 (MDR1) and MDR1 mRNA expression. The effects of miR-451 on MDR1 expression appeared to be mediated through inhibiting the expression of some transcriptional factors involved in suppressing MDR1 gene product that confers cancer cell resistance to a broad range of chemotherapeutics. The sensitivity to and intracellular accumulation of cytotoxic drugs that are transported by P-glycoprotein were enhanced by the treatment with the antagomirs of miR-451. In contrast, the mimics of miR-451 increased MDR1 expression. Their results therefore demonstrated for the first time the roles of microRNAs in the regulation of drug resistance mediated by MDR1/P-glycoprotein, and suggested the potential for targeting miR-451 as a therapeutic strategy for modulating MDR in cancer cells

Imatinib is a substrate of P-gp-mediated efflux. The up-regulation of drug transporters is one of specific causes of IM resistance, since it can be effluxed through MDR1 transporters, resulting in enhanced clearance of drug from the cell, reduced drug availability and drug resistance which suggested that miR-451 could play an important role in IM resistance.[141,142]

The above findings, therefore highlight the role of miR-451 as a key player in cancer therapeutics and specifically as a potential marker to predict response to IM treatment in CML patients.[112]

As regards CBC parameters, in the present study, there was a statistically significant reduction in both the mean hemoglobin concentration ($p< 0.001$) and the mean platelet count ($p< 0.001$) in group I (newly diagnosed CML patients) compared to group II, group III and group IV ($p< 0.001$). The mean WBC count however was significantly increased ($p< 0.001$) in newly diagnosed CML patients compared to the three other groups. CML cases in groups II and III showed no statistically significant difference in the mean values of any of their CBC parameters being studied when compared to healthy controls due to the fact that patients from groups II and III are on current treatment which lead to normalization of their CBC parameters.[30]

Statistical analysis of BCR-ABL% in this study, showed no significant difference between CML cases regarding BCR-ABL% at diagnosis, while the median value of BCR-ABL% on follow up was significantly higher in IM resistant cases than in newly diagnosed cases ($p< 0.001$) and IM responder cases ($p< 0.001$). The differences between IM responders and IM resistant cases regarding their follow up BCR-ABL% 3 months after IM treatment onset was the bases for patients' selection and allocation to their respective groups in the present study.[30]

Regarding miR-451 level, results from this study showed a significant decrease in the median level of miR-451 in newly diagnosed patients (group I) compared to the healthy controls. These results are in parallel with the results of microarray analysis conducted by

Machová et al 2011,[100] who reported the down-regulation of miR-451 among several other studied miRNAs in CML. The down-regulation of miRNAs as reported by the study, may contribute to the increased levels Phosphatidylinositol-3-kinase/Protein kinase B/Mammalian target of rapamycin. (PI3K/AKT/mTOR) signaling pathway and it may potentially regulate resistance development in CML. AKT1 is a member of the anti-apoptotic pathway of PI3K that is associated with the BCR-ABL directed transformation and with the response to the TKI treatment. The PI3K/AKT/mTOR pathway is activated in IM naive cells, but under IM treatment, it may contribute to the resistant phenotype.

However, inconsistent with the present results, Xiong Q et al 2014,[139] who employed miRNA sequencing technique, reported miR-451 among several other up-regulated miRNAs in K562 cell line. In addition, there were other up-regulated miRNAs including four members of the polycistronic miR-17-92 cluster that were previously reported to be involved in CML pathogenesis.[97] Moreover, the study also reported an up-regulation of miRNA (10a, 125, 151, 199a-5p, 96, 451, 183, 134, 126, 144 and 224) which had all been previously detected in CML patients.[31,138] Nevertheless, the study also suggested a dynamic nature for miRNA expression at different stages of myeloid development.

In the current work, newly diagnosed cases showed the lowest median miR-451 among CML cases. Also, there was a significant increase in the median of miR-451 level in IM responder compared to IM resistant cases ($p=0.046$). Moreover, miR-451 was down-regulated in leucocytes of 80% of the newly diagnosed untreated patients, 26.7 % of IM responder patients and 33.3% of the IM resistant cases while it was up-regulated in 20%, 73.3% and 66.7% in the respective groups.

These findings were supported by those reported by Lopotová et al 2011,[128] who analyzed miR-451 expression in total leukocytes of CML patients at the time of diagnosis, in major molecular response, in hematological relapse, in suboptimal response and in healthy controls using qPCR. By this approach, they found miR-451 down-regulation in most of the newly diagnosed cases, increased levels in IM responders and IM resistant cases, which is in accordance with the present results.

In this regard, Scholl et al 2012,[129] conducted a retrospective study and showed that miR-451 was down-regulated in the diagnostic samples of the IM resistant group when compared with the IM responder group. Because CML patients with cytogenetic response (CCyR) might present heterogeneity in respect of their molecular responses during TKI therapy, they also analyzed the miR-451 expression among cytogenetic responders. No significant differences in the miR-451 expression were observed between patients with CCyR when discriminated by the depth of their molecular responses. This suggested that the predictive value of miR-451 levels at diagnosis in respect to the achievement of a CCyR with IM therapy is not extended to the molecular level.

Moreover, these findings were in agreement with Liu et al 2012,[119] who identified that miR-144/451 cluster expression was significantly repressed in IM resistant cells, which might be due to the increased expression of cyto-myelocytomatosis (c-myc). They also reported that restoration of miR-144/451 could sensitize the IM resistant cells to apoptosis. Thus, miR-451 can sustain IM resistance by targeting critical signal transducers.

In the present work, statistical analysis showed no significant relation between miR-451 and age below and above 50 years (the age of highest incidence of CML) in total cases (p= 0.417). However, this finding is not in agreement with the results reported by Hooten et al 2010,[143] who studied miRNA expression alteration with age in humans. They found that most miRNAs were down-regulated in older participants compared to younger participants. Nonetheless, no specific data regarding miR-451 were reported by their study.

Several prognostic scoring systems have been developed for patients with CML, of which the Sokal and Hasford scores are the most popular. Both of these scoring systems stratify patients into three risk groups (low, intermediate, and high). The Sokal score was generated using chronic phase CML patients treated with busulphan or hydroxyurea and have also been used for the risk stratifications of patients in clinical trials evaluating TKIs.[22,23] Sokal score in the present work showed no statistically significant difference between the studied groups (p= 0.716), also no statistically significant relation between Sokal score and miR-451 was shown (p= 0.603). Flamant et al 2010,[101] however noted a significant correlation between the Sokal score and miR-142-3p levels in pre-treatment patient samples.

In the current study, the statistical relation between miR-451 expression with BCR-ABL% was significant at diagnosis in group I cases (p= 0.021), while no significant relation was shown with BCR-ABL % on follow up of this group 3 months after treatment onset (p= 0.060). However, miR-451 showed a significant inverse relation to follow up BCR-ABL % (p= 0.003) and (p= 0.002) in IM responder (group II) and IM resistant cases (group III) respectively, while no significant relation was shown with BCR-ABL % at diagnosis in the respective groups. These finding are in line with Iraci et al 2009,[144] and Lopotová et al 2011,[128] who showed previously that miR-451 had a potential to target BCR-ABL in CML and that the two parameters are inversely related.

In the present work, IM responders showed a significant negative correlation between miR-451 BCR-ABL% on follow up (p= 0.032). This finding is supported by Scholl et al 2012,[129] who reported an inverse correlation between BCR-ABL and miR-451 in IM Responder CML patients.

In accordance with the present results, the microarray study done by Polakova et al 2008,[145] reported that BCR-ABL transcript levels correlated inversely with miR-451 expression in samples of newly diagnosed cases, and IM responders, but no correlation was found in IM resistant cases (BCR-ABL as well as miR-451 levels were high in IM resistant cases). This discrepancy was also shown by their western blot analyses results, which reported a difference in BCR-ABL activity between newly diagnosed, IM responder patients and IM resistant patients.

Moreover, Lopotová et al 2011,[128] conducted in vitro cultivations of cells with IM. Interestingly, miR-451 was increased in Ph+ samples after cultivation with IM suggesting the relation of miR-451 down-regulation to BCR-ABL activity in these cells. This study suggested the possible existence of a reciprocal regulatory loop between BCR-ABL and miR-451 as a maintenance mechanism of the leukemic state of CML cells. The authors also reported that down-regulation of miR-451 might be inversely related to BCR-ABL kinase activity in CML cells.

The Receiver-Operating Characteristic (ROC) curve analysis applied in the current study to assess the performance of miR-451 as a prognostic marker for CML, estimated the sensitivity of miR-451 in detecting CML to be 42.11% while its specificity has been shown to be 80.77%, at a cut-off value of 2.51.

To sum up, data from this study indicated that the miR-451 expression is down-regulated in CML patients and that it is related to BCR-ABL kinase activity. Combination of all of the above results with the findings from previous studies, makes it evident that miR-451 serves as a prognostic tool in CML and that it plays a key role in IM resistance via its relation with BCR-ABL. Improvement of CML therapy may therefore be achieved via disrupting this relation. The results of this study can therefore serve as a reference for future laboratory and clinical studies concerning miRNAs in CML.

SUMMARY

Chronic myeloid leukemia (CML) represents 15% of adult leukemias. Imatinib Mesylate (IM) is the gold standard treatment for new cases of CML. Treatment with IM resulted in improvement of the majority of cases.

Despite the success achieved by IM in the treatment of the disease, a considerable percentage (about 25%) of cases may develop resistance to the drug. Sensitive and specific early predictors of IM resistance in CML patients have not been established to date.

The aim of the present work was to study the possible value of microRNA 451 (miR-451) in CML as an early predictor for IM resistance in Egyptian patients.

In order to achieve this goal, 60 subjects were included in the study and were divided into four groups; Group I included 15 chronic phase (newly diagnosed) adult CML patients group II included 15 IM responder CML patients. The study also included 15 IM resistant CML patients (Group III) and 15 healthy subjects as controls (Group IV).

All CML patients were subjected to:

- Thorough clinical evaluation focusing on symptoms and signs of CML.
- Real time PCR was performed to detect BCR ABL gene mutation in CML patients.

All Subjects were subjected to the following investigations:

- Routine laboratory investigations including:
  1. Complete blood count (CBC).
  2. Liver function tests including serum alanine aminotransferase (ALT) and serum aspartate aminotransferase (AST).
  3. Kidney function tests.

- Measurement of leucocytic level of micro-RNA 451 using qPCR.

Statistical analysis of the studied parameters showed the following results:

- There was a significant decrease in the median miR-451 in group I compared with groups II, III, and the control group IV. Also, there was a significant increase in the median miR-451 in group II compared with group III and the control group IV. However, group III showed a significant increase in the median miR-451 in comparison with the control group IV.

- In the comparison between the studied groups according to miR-451 expression, down-regulation of miR-451 was shown in leucocytes of 12, 4 and 5 patients in groups I, II and III respectively while it was up-regulated in 3, 11 and 10 patients in groups I, II and III respectively.

- Regarding the relation between miR-451 and BCR-ABL % in group I, a significant relation was shown at diagnosis while no significant relation was shown on follow up. On the other hand, in group II and group III the relation between miR-451 and BCR-

ABL % was significant on follow up while no significant relation was shown at diagnosis.

- Statistical correlations between miR-451 and other studied parameters in patients of the different groups showed a significant positive correlation between miR-451 and platelet count and a significant negative correlation with BCR-ABL% at diagnosis in the (newly diagnosed cases) group I. In (IM responder cases) group II a significant negative correlation between miR-451 with BCR-ABL% on follow up was shown. However, (IM resistant cases) group III showed no significant correlations between miR-451 and any of the studied parameters.
- The Receiver-Operating Characteristic (ROC) curve analysis applied to assess the performance of miR-451 as a prognostic marker for CML revealed that at the cut-off value of 2.51 the sensitivity of miR-451 in detecting CML has been estimated to be 42.11% while its specificity has been shown to be 80.77%.

CONCLUSION

1. MicroRNA 451 (miR-451) expression was significantly decreased in newly diagnosed chronic myeloid leukemia (CML) patients as compared to healthy subjects.

2. MicroRNA 451expression was significantly increased in Imatinib Mesylate (IM) responder CML patients compared with IM resistant CML, newly diagnosed CML patients and healthy controls.

3. At the cut-off value of 2.51 the sensitivity of miR-451in detecting CML has been estimated to be 42.11% while its specificity has been shown to be 80.77%. Therefore, it could be used as a useful additional follow up marker for the response to IM and as a promising prognostic biomarker for CML.